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14. ABSTRACT
The COE investigators made outstanding progress in the third year. We found that we can analyze 40 metabolites, conjugates and depurinating DNA adducts of estrogens in the urine and serum of women. The women with breast cancer had high levels of depurinating adducts in the urine, whereas the control women had baseline levels. For the first time, it was demonstrated that E2 induces complete transformation of human epithelial cells with formation of tumors in SCID mice. Furthermore, the ability to characterize cell transformation at the combined levels of the complete genome and the individual gene was determined. The lac I rats showed low mutagenic activity of the mammary tissue when treated with 4-OHE2 and no mutagenicity after treatment with 2-OHE2. The ERKO mice without estrogen receptors, E2 induced tumors in mammary tissue and produced genotoxic metabolites. All these findings provide strong support that estrogens can become genotoxic compounds and eventually initiate breast cancer.

15. SUBJECT TERMS Estrogens, estrogen-initiated cancer, depurinating estrogen-DNA adducts, analysis of estrogen metabolites and conjugates, estrogen-induced mutation and cell transformation, overexpression of aromatase, gene expression by DNA microarray, human breast ductal lavage, breast cancer prevention, biomarkers for breast cancer susceptibility

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SPECIFIC AIM 1 – CAVALIERI

A.   Introduction
        The basic hypothesis guiding this research is that endogenous estrogens can be oxidatively metabolized to catechol estrogen quinones that react with DNA to form specific DNA adducts to generate tumor-initiating mutations. These DNA adducts are potential biomarkers for breast cancer and risk of developing breast cancer. In fact, the level of the 4-hydroxyestrone(estradiol)-1-N3Adenine adduct [4-OHE$_1$(E$_2$)-1-N3Ade] has been observed to be significantly higher in urine from men with prostate cancer or other urological conditions than in urine from healthy control men, in which the adduct is at background levels [1]. The results obtained in animal models, cell culture and human breast tissue led us to select several compounds to prevent the genotoxicity of estrogens that we think is at the origin of breast cancer. The selected compounds target different steps involved in the mechanism of tumor initiation. Prevention studies will demonstrate that estrogen genotoxicity plays a critical role in the initiation of breast cancer. In addition, the results will lay the groundwork for designing a clinical research study of breast cancer prevention and developing bioassays for susceptibility to this disease. With these goals in mind, we are searching for an animal model for estrogen-initiated cancer, particularly breast cancer, and we are developing data to demonstrate the utility of the depurinating estrogen-DNA adducts as biomarkers for breast cancer.

B.   Body

B-i.   Methods and Procedures
        In Specific Aim 1a, neonatal Sprague-Dawley rats were injected with 10 µg of E$_2$, 2-OHE$_2$, 4-OHE$_2$ (or solvent)/pup/day on days 1-5 of life [2]. The rats were weaned, divided by sex, observed for 1 year, sacrificed, and examined for tumors, especially mammary tumors in the females. A second experiment was planned to begin to study the ability of selected natural compounds to prevent the initiation of tumors by estrogens.

        In Specific Aim 1b, progress was made in analyzing nipple aspirate fluid from women with and without breast cancer by using ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS). We also began analyzing urine samples from women with and without breast cancer and serum samples from women without breast cancer (samples were provided or collected under other IRB protocols).

B-ii.   Results
Specific aim #1a: Investigate the prevention of estradiol (E$_2$)-induced tumors in the mammary gland of female ACI rats by analyzing the profiles of estrogen metabolites, conjugates and depurinating DNA adducts in treated animals at various time-points and the development of tumors in the animals.
Neonatal Initiation of Mammary or Prostate Tumors in Sprague-Dawley Rats by Catechol Estrogens
        Twenty pregnant Sprague-Dawley rats were shipped from Harlan Teklad at 13 days of gestation and caged individually. At delivery the rats' pups were given daily i.p. injections of estrogens at 10 µg/pup/day in 10 µL of trioctanoin-DMSO (95:5) with a 30g needle on days 1- 5 of neonatal life [2]. [The injections were of 10 µg E$_2$, 2-OHE$_2$ or 4-OHE$_2$/pup/day. The rats in the control groups received only 10 µL of trioctanoin-DMSO (95:5)]. The average weight of a neonatal rat is 5 -7 g. The pups were weaned at 21 days of age, the dams euthanized, the litters

combined by sex and treatment and housed five animals/cage. The rats were monitored by recording body weights and palpating the mammary glands of the females. Twelve months after the neonatal injections, surviving rats were euthanized and complete necropsies were performed. Skin panels containing the mammary glands were excised from the females and the urogenital tract was excised from the males and fixed in 10% buffered formalin as well as selected internal organs.

The treatment group sizes were:

Compound	Females	Males
E_2	42	35
$2\text{-}OHE_2$	34	40
$4\text{-}OHE_2$	28	38
Solvent control	33	31

Neither the female rats nor the male rats developed any tumors during this experiment. Presumably, this is because the Sprague-Dawley strain is truly refractory to estrogen-initiated tumors. Therefore, Sprague-Dawley rats cannot be used as a model for breast cancer.

<u>The Effect of Dietary *N*-Acetylcysteine on Neonatal Initiation of Uterine Adenocarcinomas in Female CD-1 mice by Catechol Estrogens</u>
To begin to study the ability of selected natural compounds to prevent estrogen-initiated cancers, we planned to study the effect of *N*-acetylcysteine (NAcCys) on the initiation of uterine adenocarcinomas by E_2, $2\text{-}OHE_2$ or $4\text{-}OHE_2$ [2]. We planned to have 70 pregnant CD-1 mice shipped to us at 13 days of gestation and fed diet containing one of two levels of NAcCys grain-based chow through delivery and weaning of the pups. At delivery the mouse pups would be given daily i.p. injections of E_2, $2\text{-}OHE_2$ or $4\text{-}OHE_2$ (or solvent) at 2 µg/pup/day in 10 µL of trioctanoin-DMSO (95:5) with a 30g needle on days 1- 5 of neonatal life. (The average weight of a neonatal mouse is $1 - 2$g.) The pups would be weaned at 21 days of age and fed the dam's level of NAcCys chow; body weights and food consumption would be recorded at regular intervals and any morphological changes charted. Twelve months after the neonatal injections the surviving mice would be euthanized, complete necropsies performed, the uteruses weighed and the internal organs fixed in 10% buffered formalin Tumors would be histopathologically examined.

As we finalized plans for this experiment, we realized that before weaning, the mouse pups would ingest the preventive agent only through the dams' milk. Those 21 days would be critical times for prevention of cancer initiation. Therefore, after considerable discussion, we concluded that this experimental design was not appropriate for testing the ability of a compound to prevent estrogen-initiated cancer, and we did not conduct the experiment.

Now that we have detected relatively high levels of depurinating estrogen-DNA adducts in urine samples from women with breast cancer (see below), we will be able to study the effects of potential preventive agents on adduct levels in humans.

Specific Aim #1b: Analyze the profiles of estrogen metabolites, conjugates and depurinating DNA adducts in ductal lavage samples from women with and without breast cancer.

We have progressed in analyzing nipple aspirate fluid samples from women with and without breast cancer for 36 estrogen metabolites, estrogen conjugates and depurinating estrogen-DNA adducts by UPLC/MS/MS (Table 1). In the small number of samples analyzed, we have detected the depurinating DNA adducts 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1-N7Gua in nipple aspirate fluid from women with breast cancer, but not in nipple aspirate fluid from women without breast cancer. We are currently collecting more samples for analysis, so that we can determine whether this difference will hold up in a larger population.

Collection of nipple aspirate fluid has presented problems and our success rate is approximately 50%. The samples are generally small (perhaps 10 µl) and can be difficult to recover from the capillary tubes in which they are collected and stored at −80 °C. As we proceed, we hope to overcome these problems.

Table 1. Estrogen-DNA adducts in nipple aspirate fluid from women with and without breast cancer

Subjects	$\dfrac{\text{4-hydroxy estrogen-DNA adducts}}{\text{4-hydroxy estrogen compounds}^{a}}$ X 10^3		
	N3Ade	N7Gua	Total
Women with breast cancer			
1	17	183	200
2	64	0	64
3	91	0	91
4	70	59	129
Women without breast cancer			
1	0	0	0
2	0	0	0

In addition, we have begun analyzing urine samples (spot samples of approximately 50 ml) from women with and without breast cancer for 36 estrogen metabolites, estrogen conjugates and estrogen-DNA adducts (Table 2). In the small number of samples analyzed thus far, the level of estrogen-DNA adducts, both 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1-N7Gua, is significantly greater in urine from women with breast cancer than in urine from healthy control women. These results suggest that the estrogen-DNA adducts can be used as biomarkers for breast cancer. If the formation of estrogen-DNA adducts is the first critical event in the initiation of breast cancer, as we hypothesize, then these adducts could used as biomarkers for early diagnosis of breast cancer risk. These adducts also will serve as surrogate endpoint biomarkers in studies of potential agents to prevent breast cancer.

We have also developed a preparative procedure and begun to analyze serum samples from women without breast cancer (healthy control women). We have found low levels of 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1-N7Gua in these samples.

B-iii. Proposed Research for the Next Year

1. Specific Aim #1a:

Although we did not detect tumors in the Sprague-Dawley rats treated as neonates with E_2, 2-OHE$_2$ or 4-OHE$_2$, we will have selected tissues examined histopathologically to make sure that there is no evidence of tumor formation. We have no ideas for other possible animal models for demonstrating the initiation of breast cancer by estrogens.

Table 2. Excretion of estrogen-DNA adducts in urine by women with and without breast cancer[a]

		$\dfrac{\text{4-hydroxy estrogen-DNA adducts}}{\text{4-hydroxy estrogen compounds}^{b}}$ X 10^3					
Controls[c]	N3Ade	N7Gua	Total	Cases	N3Ade	N7Gua	Total
1 − 1	0.5	1.4	1.9	1[d]	131	167	298
1 − 2	0.6	0.9	1.5				
2 − 1	1.0	2.9	3.9	2	47	120	167
2 − 2	0.5	2.4	2.9	3	38	174	212
3 − 1	0.6	1.2	1.8				
3 − 2	0.9	0.9	1.8	4[e]	706	116	822
4 − 1	0.8	0.8	1.6	5	28	21	49

[a]In 2-ml aliquots of urine, 30 estrogen metabolites and estrogen conjugates plus 6 depurinating estrogen-DNA adducts were analyzed by ultra-performance liquid chromatography/tandem mass spectrometry.
[b]These are the 4-catechol estrogens + 4-methoxycatechol estrogens + 4-catechol estrogen-GSH conjugates.
[c]Control subjects provided 2 urine samples 1 week apart, except for #4.
[d]Possible recurrent breast cancer.
[e]Recurrent breast cancer.

2. Specific aim #1b:

In the coming year, we will continue to collect and analyze nipple aspirate samples from women with and without breast cancer by UPLC/MS/MS. We expect to have sufficient samples to demonstrate a statistically significant difference in the levels of estrogen-DNA adducts in breast fluid from women with and without breast cancer.

In addition, we will continue to analyze urine samples from women with and without breast cancer for estrogen metabolites, estrogen conjugates and estrogen-DNA adducts. It will be very interesting to discover whether the levels of estrogen-DNA adducts in one body fluid (nipple aspirate fluid, urine and serum) are predictive of the levels in the other fluids or that they are complementary to the levels in the other fluids. We will learn which of these fluids provides the most useful results or if there is value in analyzing more than one of them.

We have recently received permission to collect serum samples from women with breast cancer (protocol not supported by the COE), and we plan to collect and analyze these samples for the 36 estrogen metabolites, estrogen conjugates and estrogen-DNA adducts. We anticipate finding that the levels of the adducts are significantly higher in serum from women with breast cancer, compared to serum from the healthy control women.

**C.	Key Research Accomplishments**
1.	We have concluded that the Sprague-Dawley rat is not a model for estrogen-initiated tumors.
2.	We have found in a small set of samples that nipple aspirate fluid from women with breast cancer contains estrogen metabolites, conjugates and depurinating DNA adducts, but nipple aspirate fluid from healthy control women does not contain the DNA adducts at levels detectable by UPLC/MS/MS. The adducts 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1-N7Gua in nipple aspirate fluid are potential biomarkers for breast cancer.
3.	We have demonstrated that urine from women with breast cancer contains significantly higher levels of 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1-N7Gua than does urine from healthy control women. The adducts 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1-N7Gua in urine are potential biomarkers for breast cancer.
4.	We have demonstrated that serum from women without breast cancer (healthy control women) contains baseline levels of estrogen metabolites, estrogen conjugates and the estrogen-DNA adducts 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1-N7Gua. The adducts 4-OHE$_1$(E$_2$)-1-N3Ade and 4-OHE$_1$(E$_2$)-1-N7Gua in serum are potential biomarkers for breast cancer.

**D.	Reportable Outcomes**

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Rogan, E. Invited talk: Xenoestrogens, Biotransformation, and Differential Risks for Breast Cancer, The 13th International Symposium on Functional Medicine, Managing Biotransformation: The Metabolic, Genomic, and Detoxification Balance Points, Tampa, FL, April 19-22, 2006.

E.   Conclusions

1.   We have not been successful in finding an animal model for estrogen-initiated mammary tumors.

2.   We have detected estrogen metabolites, GSH conjugates and depurinating DNA adducts in our initial studies of nipple aspirate fluid. The adducts, $4\text{-OHE}_1(E_2)\text{-1-N3Ade}$ and $4\text{-OHE}_1(E_2)\text{-1-N7Gua}$, were detected in nipple aspirate fluid from women with breast cancer, but not from women without breast cancer.

3.   We have detected estrogen metabolites, GSH conjugates and depurinating DNA adducts in our initial studies of urine. The adducts, $4\text{-OHE}_1(E_2)\text{-1-N3Ade}$ and $4\text{-OHE}_1(E_2)\text{-1-N7Gua}$, were detected at significantly higher levels in urine from women with breast cancer, compared to urine from women without breast cancer. These adducts are potential biomarkers for breast cancer.

4.   We have detected estrogen metabolites, GSH conjugates and depurinating DNA adducts in our initial studies of serum. The adducts, $4\text{-OHE}_1(E_2)\text{-1-N3Ade}$ and $4\text{-OHE}_1(E_2)\text{-1-N7Gua}$, were detected at baseline levels in serum from women without breast cancer. These adducts are potential biomarkers for breast cancer.

5.   In our hypothesis, estrogen-3,4-quinones are initiators not only of breast cancer, but also of other types of human cancer, for example, prostate, non-Hodgkin's lymphoma, ovary, etc. Therefore, we hypothesize that higher levels of estrogen-DNA adducts in urine would be biomarkers for all of these cancers. Detection of depurinating estrogen-DNA adducts in nipple aspirate fluid would be a specific test for breast cancer.

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SPECIFIC AIM 2 - RUSSO

A. INTRODUCTION

Breast cancer is a malignancy whose dependence on ovarian function was first recognized through the regression of both advanced cancer (1) and metastatic disease (2) induced by oophorectomy in premenopausal women. Ulterior correlation of ovarian function with estrogen production (3), and the isolation of the estrogen receptor protein (4,5), combined with the observed greater incidence of estrogen receptor positive tumors in postmenopausal women (6-16), led to the identification of a strong association of estrogen dose and length of exposure with increased breast cancer risk (6,10,13,14). The importance of ovarian steroidogenesis in normal breast development and in the genesis of breast cancer is highlighted by the facts that early menarche and late menopause are associated with greater breast cancer risk, whereas late menarche and early menopause, that occurring before 40 years of age, result in a significant reduction of the same (17-20). Breast development at puberty and during sexual maturity is stimulated by 17β-estradiol (E_2), which is the predominant circulating ovarian steroid and the most biologically active hormone in breast tissue (21,22). At menopause E_2 plasma levels decrease by 90% (17-19). In spite of the markedly different circulating levels of estrogens in pre- and postmenopausal women, the concentrations of E_2 in breast cancer tissues do not differ between these two groups of women, an indication that its uptake from the circulation might not contribute significantly to the total content of this hormone in breast tumors, but rather that *de novo* biosynthesis, i.e., peripheral aromatization of ovarian and adrenal androgens, plays a more significant role (23,24).

Considerable epidemiological and clinical evidence link cumulative and sustained exposure to estrogens with increased risk of developing breast cancer. However, there is no clear understanding of the mechanisms through which estrogens cause cancer. In experimental animal models it has been demonstrated that E_2, 11β-methoxyethinylestradiol (Moxestrol), and diethylstilbestrol (DES), as well as their 4-hydroxycatechols, induce kidney cancer in castrated male Syrian golden hamsters (25-27). In rats, continuous administration of supraphysiological doses of estrogens induces a high percentage of mammary adenocarcinomas, whereas low doses given over long periods induce fibroadenomas (28). In both models, however, the tumorigenic effects of estrogens are associated with marked hyperprolactinemia and pituitary hyperplasia resulting from an increase in number of hyperplastic prolactin secreting cells. The dependence on a functional pituitary gland has been further confirmed in hypophysectomized rats in which estrogens are ineffective as carcinogens (29). Nevertheless, the most widely acknowledged mechanism of estrogen carcinogenicity is its binding to its specific nuclear receptor alpha (ER-α) for exerting a potent stimulus on breast cell proliferation through its direct and/or indirect actions on the enhanced production of growth factors (21,22). However, the fact that ER-α knockout mice expressing the Wnt-1 oncogene (ERKO/Wnt-1) develop mammary tumors provides direct evidence that estrogens may cause breast cancer through a genotoxic, non-ER-α-mediated mechanism (30,31). This postulate is further supported by the observations that when ovariectomized mice are supplemented with E_2 they develop a higher tumor incidence with shorter latency time than controls, even in the presence of the pure antiestrogen ICI-182,780. Experimental studies on estrogen metabolism (32,33), formation of DNA adducts (34), carcinogenicity (35-37), mutagenicity (38), and cell transformation (39-42) have supported the hypothesis that reaction of specific estrogen metabolites, namely, catechol estrogen-3,4-quinones (CE-3,4-Q) and to a much lesser extent, CE-2,3-Q, can generate critical DNA mutations that initiate breast, prostate and other cancers (43). In order to definitively outline the pathways

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through which estrogens act as carcinogens in the human breast and for assessing whether one or more of the mechanisms described above are responsible of carcinogenic initiation, it is needed an experimental system in which E_2 by itself or its metabolites induce transformation of human breast epithelial cells (HBEC) in a well controlled environment, preferentially *in vitro.* Towards this purpose we have developed an *in vitro/in vivo* system of cell transformation that fulfills these requirements. Using this model we have demonstrated that E_2 and its metabolite 4-hydroxyestradiol (4-OH-E_2) induce transformation of MCF-10F, an ER-α negative human breast epithelial cell line (39-42, 44). In response to estrogen treatment the cells form colonies in agar methocel, lose the capacity to differentiate by forming three-dimensional structures when grown in a collagen matrix, or their ductulogenic capacity, forming instead spherical and solid masses, and exhibit an increase in cell proliferation and in their invasive capabilities in Matrigel (39-42,44). More importantly, the expression of these phenotypes indicative of neoplastic transformation was not abrogated by their simultaneous treatment with the anti estrogen ICI-182,780 (ICI), suggesting that the transformation of MCF-10F cells by these compounds did not require the presence of the ER-α (40,41). The present work describes the novel findings that in this experimental model, E_2-induced transformation of HBEC *in vitro* increased the invasive potential of the cells. In addition, the selection of the most highly invasive cells in the Matrigel chambers identified transformed cells that express phenotypic and genotypic variations that correlate with their tumorigenic potential in a heterologous host, but still maintained their cell lineage characteristics. We also report that the induced tumors exhibit genomic alterations that are similar to those reported in primary breast cancer, as determined by comparative genomic hybridization (CGH).

B. BODY

B-i- Methods and procedures.

B-i-a- *MCF-10F cells transformed with17-β estradiol treatment*
These studies were performed utilizing the following cell lines: the spontaneously immortalized estrogen receptor alpha (ER-α) negative human breast epithelial cell line MCF-10F, which was cultured in DMEM:F12 medium containing 1.05mM calcium, antibiotics, antimycotics, hormones, growth factors, and equine serum as previously described (44,45). MCF-10F cells in their 123rd passage were treated with 70nM 17β-estradiol (E_2) (Sigma Co, St. Louis, MO) dissolved in DMSO. This dose was selected because it induced greater colony efficiency in agar methocel, number of solid masses in collagen gel, and invasiveness in the Matrigel invasion chamber than the 0.007nM dose of E_2 previously tested (39,40,44). E_2 was added to the culture medium for 24 hours at 72 and 120 hours post-plating. At the end of the first week of treatment, the cells were passaged for administration of another two 24 hour-periods of E_2 treatment (39). At the end of each treatment period, the culture medium was replaced with fresh medium. MCF-10F cells treated with DMSO at a final concentration of 0.01% were used as controls. All cells were collected 24 hours after the last treatment, and thereafter they were maintained in culture for ten additional passages.

B-i-b- *Selection of E_2-treated MCF-10F cells by Matrigel invasion assay*
Control and E_2-treated MCF-10F cells in their 10th passage were trypsinized and seeded in the upper chamber of seven and eight Matrigel invasion chambers, respectively, at a concentration of 2.5 x 10^4 cells/well each; they were incubated at 37 °C in a 5% carbon dioxide incubator for 22 hours. At the end of this period, the inserts of each chamber were carefully

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removed from the wells with sterile forceps and then the upper/inner surface of the membranes were wiped with cotton tipped applicators for removing all non-invading cells. Each membrane was then cut from the insert using a sterile scalpel blade and individually placed in a well from a 24-well plate, with the lower surface that held the invading cells up, facing the culture medium. The cells were fed with DMEM medium containing 5% horse serum, maintained at 37 °C in a 5% CO_2 incubator until they reached confluence, and then transferred to a 75cc flask. Seven cell lines from control MCF-10F cells were thus selected and labeled A-1 to A-7. Four cell lines were selected from E_2–treated MCF-10F cells, which were designated B2, C3, C4, and C5 (Fig. 1).

B-i-c- *Evaluation of transformation phenotypes*

Control MCF-10F cells and E_2-transformed cells at passage 10, and those invasive cell lines isolated from the invasion chamber described above were expanded and evaluated for the expression of the following phenotypes of neoplastic transformation: colony formation in agar-methocel, or colony efficiency (CE), ductulogenic capacity in collagen-matrix, invasiveness in Matrigel invasion chambers, and tumorigenic assay in severe combined immunodeficient (SCID) mice.

MDA-MB-231, an ER-α negative human breast cancer cell lines purchased from the Tissue Culture Collection (Rockville, MD) and BP1-Tras, a tumorigenic cell line derived from benz(a)pyrene (BP)-transformed-c-Ha-ras transfected MCF-10F cells (46) were used as positive controls.
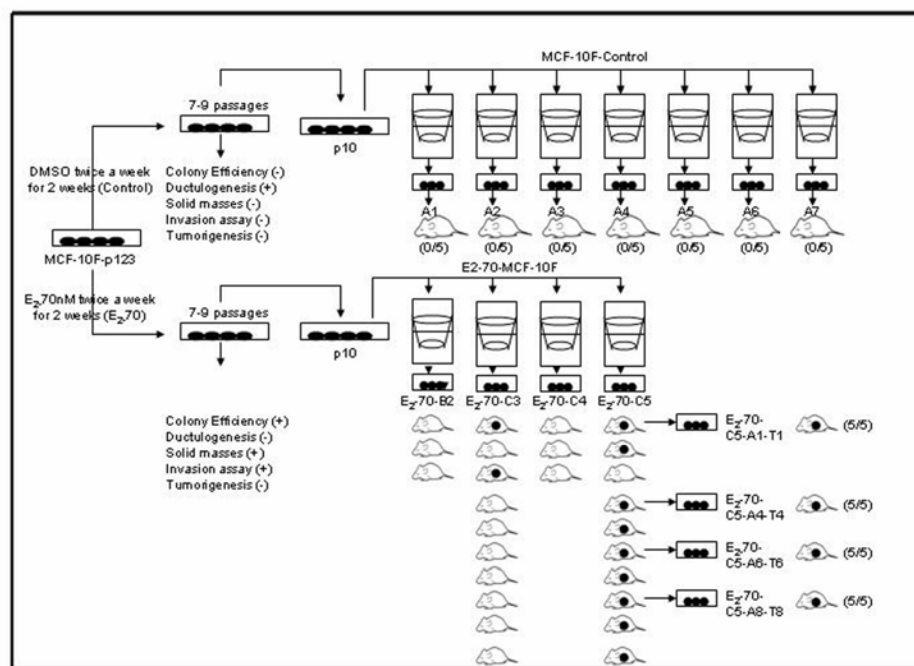

Figure 1: Schematic representation of the experimental protocol. MCF-10F cells in their 123rd passage were treated with 70nM 17 β-estradiol (E_2-70) or DMSO (Control) for 24 hrs periods twice a week for 2 weeks; 24 hrs after the last treatment the cells were plated, grown to confluence and passaged for 7-9 times before being tested for colony efficiency, ductulogenesis in collagen, solid mass formation, invasion assay in Matrigel and tumorigenesis in SCID mice. At the 10th passage the cells were trypsinized and seeded in the insert of Matrigel invasion chambers at a concentration of 2.5×10^4 cells/well, incubated for 22 hours and then the membranes of the inserts were cut and invasive cells were cultured in 24-well plates. MCF-10F Control cells generated seven cell lines that were designated A1 to A-7, and E_2-70-treated cells four cell lines, designated B2, C3, C4, and C5. All invasive cells were expanded and evaluated for the expression of tumorigenesis in SCID mice. None of the animals injected with MCF-10F Control cells or with E_2-70-B2 and E_2-70-C4 cells developed tumors. Two out of ten and nine out of ten mice injected with E_2-70-C3 and E_2-70-C5 cells, respectively, developed tumors. From these latter, tumors from four of the animals were dissected in 0.5-1mm size fragments, incubated in culture medium until confluent, generating cell lines from each tumor that were subsequently injected to 5 mice per cell line for evaluating their tumorigenic potential. All injected animals developed tumors. All tumors and cell lines were analyzed histopathologically and immunocytochemically as well as for fingerprint and CGH analyses.

B-i-d- *Colony formation in agar-methocel*

Control and treated cells were suspended at a density of 2×10^4 cells/ml in 2 ml of 0.8%

methocel (Sigma Co, St. Louis, MO) dissolved in DMEM:F-12 (1:1) medium containing 20% horse serum. Cells from each treatment group were plated in eight 24-well chambers pre-coated with 0.5 ml 5% agar base in DMEM: F-12 medium. Cells were fed with fresh medium twice a week. For evaluation of colony efficiency, the total number of viable cells was counted at 10X magnification in four wells that were stained with neutral red (1:300) after 24 hours post plating and in four additional wells after 21 days in culture. In the latter, each colony was measured using a graduated eyepiece fitted in a transmission light microscope at 10X magnification. The number of colonies greater than 50 microns in diameter was counted and results of colony efficiency were expressed as a percentage of the original number of viable cells after 24 hrs of plating.

B-i-e- *Ductulogenic assay*

Control and E_2-treated MCF-10F cells at their ninth passage post-treatment were suspended at a final density of $2x10^3$ cells/ml in 89.3% (Vitrogen^{100}) collagen matrix (Collagen Co., Palo Alto, CA) and plated into four 24 well chambers pre-coated with 89.3% of collagen base. The cells were fed with fresh medium containing 5% horse serum twice a week. The cells were examined under an inverted microscope for a period of 21 days or longer for evaluation of the number of duct-like or spherical mass structures as described in (40). At the end of the observation period the structures were photographed, fixed in 10% neutral buffered formalin and processed for histological examination.

B-i-f- *Invasion assay*

The invasion assay was performed using 24-well plate matrigel invasion chambers (BD Biosciences, Bedford, MA) fitted with cell culture inserts (Falcon Cell Culture Inserts) closed with an 8 μm pore-size PET membrane coated with a uniform Matrigel basement membrane matrix. Chambers were stored at –20 °C and brought to room temperature in a laminar flow hood for 2 hours and the insert chambers were hydrated by placing 500 μl of culture medium containing 5% horse serum at 37 °C for 2 hours in a humidified tissue culture incubator. Then the medium was removed from the inserts and 500μl of 20% horse serum were added to each well as chemoattractant. E_2-treated, and all control cells were trypsinized and each cell line was seeded in triplicate in the upper chamber at a concentration of 2.5×10^4 cells/well and incubated at 37 °C in a 5% carbon dioxide incubator for 22 hours. At the end of this period, the membranes of each chamber were fixed with Diff-Quick fixative and stained with Diff Quick Solutions I and II (Sigma Chemical Co., St. Louis, MO), cut out with a sharp scalpel and mounted onto glass slides. The total number of cells that invaded through the membrane was counted under a light microscope and the invasion index was expressed as the means ± standard error (SE) of the cells that migrated through the membrane and attached to the lower surface.

B-i-g- *Tumorigenic assay*

The tumorigenic ability of all the cell lines was tested in 45-day-old female SCID mice that were purchased from Taconic Farms (Germantown, NY). Cells were injected using protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Fox Chase Cancer Center. Trypsinized cells were suspended in PBS and injected in the mammary fat pad of the abdominal region of the mice at a concentration of $10-15x10^6$ cells in a volume of 0.1ml. The animals were palpated twice a week for detection of tumor development. Tumors were measured in two dimensions with a Vernier caliper and when they reached a maximal diameter of 1 cm, the animals were euthanized by carbon dioxide inhalation. All of the animals were autopsied and carefully examined for identification of visceral metastases. Tumors were excised

under sterile conditions and divided in three fragments; one was fixed in 10% neutral buffered formalin (NBF) and processed for histopathological and immunocytochemical examination. A second fragment was rapidly frozen in liquid nitrogen and stored at −80 °C for future use, and a third fragment was used for cell culture. Those animals that did not develop tumors were followed up for six to ten months post injection and then euthanized and autopsied, fixing the site of injection in 10% NBF for histopathological analysis.

B-i-h- *Development of cell lines from tumors*

C5 cells injected to 10 mice gave origin to 9 tumors from which 4 tumors were used for developing cell lines. The tumors were dissected in small fragments of 0.5 to 1mm in thickness and incubated in a Petri dish with culture medium until confluent, at approximately five days after plating. Explants were passed twice and maintained in DMEM culture medium with a 0.001 mM Ca^{++} concentration until confluent. The tumoral cell lines derived from C5 were designated C5-A1-T1, C5-A4-T4, C5-A6-T6 and C5-A8-T8. These cells were used at passage three for fingerprint and tumorigenic assay analyses (Fig.1).

B-i-i- *Histopathological and immunocytochemical analyses*

Tissues fixed in formalin, dehydrated, and embedded in paraffin were cut at 5 μm thickness and stained with hematoxylin and eosin for histopathological analysis. For immunocytochemical analysis, tissue sections were mounted on aminoalkylsilane-coated or positively charged slides, deparaffinized, rehydrated and incubated in 2% hydrogen peroxide at room temperature for 15 minutes for quenching endogenous peroxidase activity. The sections were sequentially incubated in two changes of Target Retrieval Solution at 98°C for 5 minutes each. All the tissue sections were incubated in diluted normal blocking serum for 20 minutes. Excess serum was blotted from the slides and the sections were incubated with the following mouse monoclonal antibodies: AE1, anti-human low molecular weight cytokeratin, AE3, anti-human high molecular weight cytokeratin, progesterone receptor clone PR88 (Biogenex, San Ramon ,CA), and anti-ER-α clone 1D5 (DakoCytomation Colorado Inc.). The polyclonal antibodies rabbit anti-human ERβ (Biogenex, San Ramon ,CA), CAM 5.2, cytokeratin peptides 7 and 8 (48 kDa and 52 kDa, respectively), and E-cadherin (Becton Dickinson Biosciences), epithelial membrane antigen (EMA) clone E29, and vimentin (DakoCytomation Colorado Inc.) were also tested. After incubation in a humidity chamber at 4 ºC overnight, sections were washed in buffer and incubated with horse biotinylated secondary antibody (Vector Laboratories, Inc., Burlingame, CA) at room temperature for 30 minutes, followed by a 30 minute incubation with Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA), washed in PBS buffer and incubated in peroxidase substrate solution containing hydrogen peroxide and 3, 3'-diaminobenzidine-HCl for 2 minutes. Sections incubated with non-immune serum were used as negative controls. All sections were lightly counterstained with hematoxylin. Immunostaining was evaluated by examination of slides under a bright field microscope, and graded according to the intensity of the brown staining.

Table 1:Markers used for fingerprint analyses.

Markers	Location	Primers (5' --3') Reverse and Forward	Ann. (Cº)
CSF1PO [Human c-fms proto-oncogene for CSF-1 receptor gene]	5q33.3-34	AACCTGAGTCTGCCAAGGACTAGC/ TTCCACACACCACTGGCCATCTTC	58º
TPOX [Introns 10 of human thyroid peroxidase gene]	2p23-2pter	ACTGGCACAGAACAGGCACTTAGG/ GGAGGAACTGGGAACCACACAGGTTA	58º
THO1 [Intron of human tyrosine hydroxylase gene]	11p15-15.5	ATTCAAAGGGTATCTGGGCTCTGG/ GTGGGCTGAAAAGCTCCCGATTAT	55º
VWA	12p12-pter	GCCCTAGTGGATGATAAGAATAATCAGTATGTG/ GGACAGATGATAAATACATAGGATGGATGG	62º
F13AO1	6p24-p25	GAG GTT GCA CTC CAG CCT TTG CAA/ TTCCTGAATCATCCCAGAGCCACA	58º
FESFPS [Human c-fes/fps proto-oncogene]	15q25-qter	GCTGTTAATTCATGTAGGGAAGGC/ GTAGTCCCAGCTACTTGGCTACTC	55º

B-i-j- *Verification of cell lineage by DNA fingerprint analysis*

Cell lineage was verified in the tumors and all the cell lines by fingerprint analysis using capillary electrophoresis and employing the markers listed in Table 1. The human breast epithelial cells MDA-MB-231 cells and BP1-Tras were used as negative and positive controls, respectively. For fingerprint analysis DNA was extracted from frozen tumors and from 70-80% confluent cells in culture (Table 2). Tissues and cells were treated with lysis buffer containing 100 mM NaCl, 20 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS and 200µg/ml proteinase K and incubated at 65ºC for 15 minutes with gentle agitation. The samples were cooled down on ice and treated with 100µg/ml RNase at 37ºC for 30 minutes. The DNA was purified with a phenol extraction (pH= 8.0) followed by chloroform: isoamyl alcohol (24:1). The aqueous layer was adjusted to 0.75M with ammonium acetate and the DNA was precipitated with 100% ethanol. The samples were centrifuged, dried and dissolved in distilled water. For fingerprint analysis, PCR was carried out in a final volume of 10 µl of 1X PCR buffer (Invitrogen) containing 1.5 mM MgCl$_2$, 0.5 pmol of each primer, 100µM dNTPs, 0.25U TaqPlatinum (Invitrogen) and 60-90ng DNA. Six markers, CSF1PO, TPOX, THO1, VWA, F13AO1 and FESFPS (Table 1) were used in this analysis. Commercially available fluorescently labeled forward primers were used in each PCR reaction. The PCR conditions consisted of a denaturation step (3min at 94ºC), followed by 35 cycles at 94ºC for 30 sec, annealing temperature for 45 sec and 72ºC for 30 sec, with an extension step at 72ºC for 5 min. The fluorescent PCR was mixed with an internal standard size marker and fractionated using CEQ8000 (Beckman Coulter). The size of the different alleles determined in the number of base pairs were compared among tumors and cell lines derived from MCF-10F with the cell line MDA-MB-231.\

We have also used variable number of tandem repeat (VNTR) analysis for confirming the MCF-10F cells lineage. For this purpose, DNA from the different cell lines was digested with HinfI and used in Southern blot analysis. The VNTR probes D2S44 and D14S13 for their corresponding markers on chromosomes 2q21.3-q22 and 14q32.1-q32.3, respectively were used.

B-i-k- *Screening for E$_2$-induced genetic changes by comparative genomic hybridization*

For determining whether the transformation of MCF-10F cells by E$_2$ treatment resulted in DNA losses and/or gains at chromosomal and subchromosomal levels we analyzed by comparative genomic hybridization (CGH) (47), which detects gains or losses of 5-15Mb, DNA obtained from control and E$_2$-treated MCF-10F cells and the invasive and tumor derived

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cells. Protocols for DNA labeling and hybridization were performed as previously described (42). Gray-level images of fluorescence were captured with a Zeiss (Thorndale, NY) microscope connected to a cooled, charge-coupled-device camera (Photometrics, Tucson, AZ). Digital image analysis was performed using the Quips software (Vysis, Downers Grove, IL). The threshold was set at 0.8 and 1.2 for losses and gains, respectively. The mean values of individual ratio profiles were calculated from at least 10 metaphase spreads. Averaged values were plotted as profiles alongside individual chromosome ideograms.

B-i-l- *Statistical analysis*

All the assays for testing colony efficiency, ductulogenesis, solid mass formation, and invasion were run in triplicate and expressed as means ± standard error (SE). The size of the tumors induced in the SCID mice was expressed as the mean of the maximum tumor diameter ± standard deviation (SD). Results were evaluated by Student's test for assessing the significance of a difference.

B-ii- Results.

B-ii- a- *Transformation of MCF-10F cells by 17-β estradiol treatment*

Treatment of the spontaneously immortalized ER-α negative human breast epithelial cell line MCF-10F with 70nM E_2 twice a week for two weeks formed colonies in agar methocel and the colony efficiency increased from 0 in controls to 12.0±1 in the treated cells (Fig. 2). The positive control cells BP1-Tras and MDA-MB231 cells had a moderately (P<0.02) and significantly (p<0.001) higher colony efficiency than E_2-transformed cells, respectively (Fig. 2).
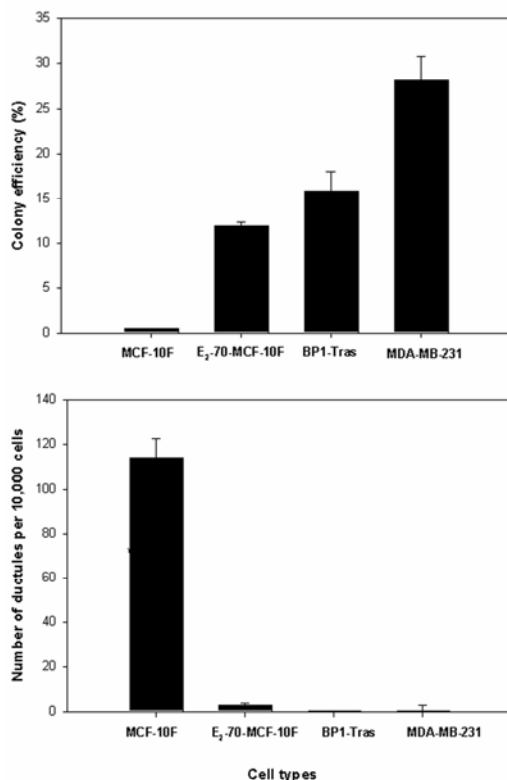

Figure 2: Anchorage independent growth, expressed as colony efficiency as described in Material and Methods. BP1-Tras and MDA-MB-231 cell lines were used as positive controls. Results are expressed as the mean ± standard error (SE) of triplicate experiments.

This treatment also affected the ductulogenic pattern of cells grown in collagen gel, which was quantitatively evaluated by counting the total number of ductules and spherical masses formed by 10,000 cells plated in collagen. Control MCF-10F cells formed an

Figure 3: Histogram depicting the ductulogenic pattern of MCF-10F cells in collagen matrix. MCF-10F control cells at 7-9 passages after DMSO treatment formed significantly higher number of ductules than E_2-70nM treated cells and than the positive controls BP1-Tras and MDA-MB-231 cells. Results are expressed as the mean ± standard error (SE) of triplicate experiments.

average of 110 ductular structures (Fig. 3), but did not form solid masses (Figs. 4 and 5). After treatment with E_2, MCF-10F cells almost completely lost their ductulogenic capacity (Figs. 3 and 5), while acquiring the ability to form spherical solid masses (Figs. 4 and 5). BP1-Tras and MDA-MB 231 exhibited a complete absence of ductule formation (Fig. 3), forming instead solid masses in

collagen gel whose values were not significantly different from those formed by E_2-treated cells (Fig. 4). The differences were highly significant (p<0.0001).
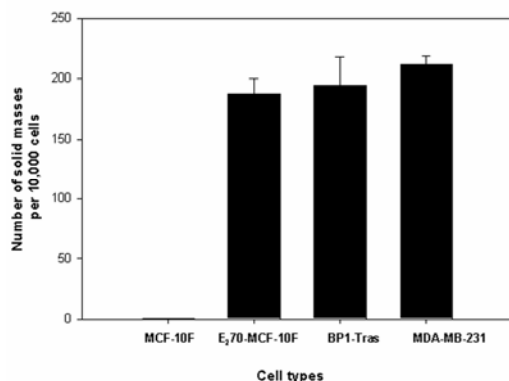

Figure 4: *Histogram depicting the formation of solid masses in collagen matrix. MCF-10F control cells at 7-9 passages after DMSO treatment did not form solid masses, whereas their number in E_2-70nM treated cells as well as in the positive controls BP1-Tras and MDA-MB-231 cells, was significantly higher. Results are expressed as the mean ± standard error (SE) of triplicate experiments.*
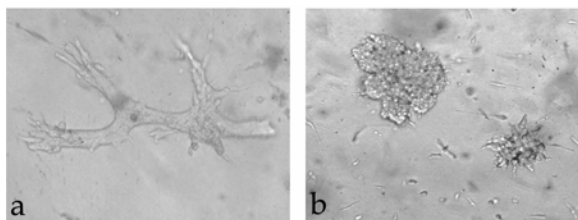

Figure 5: *a) MCF-10F cells in collagen matrix form ductules, b) MCF-10F cells transformed with 70nM of E_2 form solid masses and have lost the ability to form ductules. Phase contrast micrographs, X20)*

The ability of cells to invade a Matrigel membrane *in vitro* is a widely accepted criterion of cell transformation. Control MCF-10F cells exhibited a low invasive capacity, averaging 10±2 cells, whereas the invasive capacity of E_2-transformed cells at their 9th passage was significantly higher (80±11 cells) (Fig. 6). BP1-Tras and MDA-MB231 cells had an invasive index that was significantly higher than that of MCF-10F control and E_2 transformed cells (p<0.001).
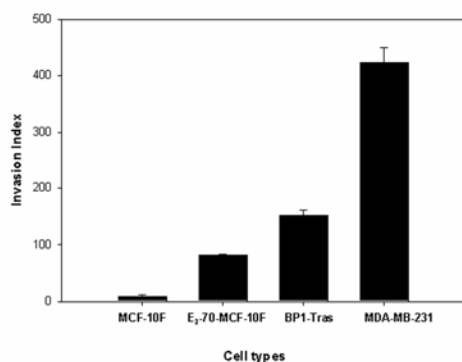
Figure 6: *Histogram depicting the invasion index of MCF-10F Control cells, $E_2$70nM transformed cells, BP1-Tras and MDA-MB-231 cells were used as positive controls. The experiments were repeated three times and results expressed as the mean ± standard error (SE).*

B-ii- b- *Tumorigenic response*

MCF-10F cells between passages 130-132 and E_2-treated cells between their passages 7 and 9 were injected to 10 SCID mice each for testing their tumorigenic capabilities. Neither control nor E_2-treated cells formed tumors after a six-month follow up period. Instead, BP1-Tras and MDA-MB231 cells were highly tumorigenic with a short latency period (Table 3). Because the tumorigenic response of these two cell lines was associated with a highly invasive phenotype, we tested whether selection of more invasive cells among E_2-transformed MCF-10F cells would allow them to express the tumorigenic phenotype, and further to determine whether this phenotype was exclusively induced by estrogen, and not the result of the selection of more

invasive control cells. For this purpose, MCF-10F cells in their 133rd passage and E$_2$-treated MCF-10F cells in their 10th passage were trypsinized and seeded in the upper chamber of seven and four matrigel invasion chambers, respectively.
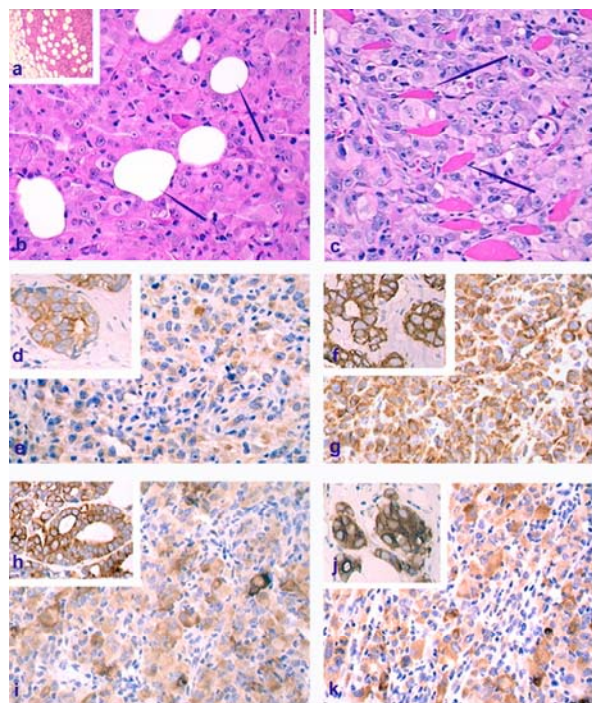

Figure 7: a: Histological section of an invasive adenocarcinoma growing in the fat pad of a SCID mouse (An 8). (H&E, 10X). b: Histological section of the invasive adenocarcinoma growing in the fat pad of a SCID mouse shown in a (An 8). Arrows point out to fat cells. (H&E, 40X). c: Histological section of an invasive adenocarcinoma growing into the muscle wall of the abdomen of a SCID mouse (An 6). Arrows indicate skeletal muscle fibers (H&E, 40X). d: Invasive ductal carcinoma of the breast immunoreacted against AE1 cytokeratin used as a positive control (40X). e: Invasive adenocarcinoma growing in the fat pad of a SCID mouse (An 8) Immunoreacted against AE1 cytokeratin (40X). f: Invasive ductal carcinoma of the breast used as positive control immunoreacted against AE3 cytokeratin (40X). g: Invasive adenocarcinoma growing in the fat pad of a SCID mouse (An 8) Immunoreacted against AE3 cytokeratin (40X). h: Invasive ductal carcinoma of the breast used as positive control immunoreacted against CAM5.2 (40X). i: Invasive adenocarcinoma growing in the fat pad of a SCID mouse (An 6) Immunoreacted against CAM5.2 (40X). j: Invasive ductal carcinoma of the breast used as positive control immunoreacted against E-cadherin (40X). k: Histological section of an invasive adenocarcinoma growing in the fat pad of a SCID mouse (An 4) immunoreacted against E-cadherin (40X).

Those cells that at 22 hours post-seeding had crossed the Matrigel membrane were cultured, giving origin to seven MCF-10F cell lines that were labeled A1 to A-7. From the E$_2$–treated cells four lines were obtained, and were designated B2, C3, C4, and C5 (Fig.1). Injection of A1 to A7 cells to SCID mice did not induce a tumorigenic response even after six months of follow up (Table 3). After injection of the E$_2$-transformed cells B2, C3, C4, and C5 to SCID mice, only C3 and C5 were tumorigenic in 2/12 and 9/10 animals injected, respectively. The clone C5 produced tumors that were larger than the ones produced by C3 (Table 3). From the 9 tumors obtained from C5 cells, four tumoral cell lines, designated C5-A1-T1, C5-A4-T4, C5-A6-T6 and C5-A8-T8 were derived. These cells were subsequently injected to another set of five SCID mice per cell line for testing their tumorigenic capabilities. All these cell lines formed palpable tumors, being C5-A8-T8 the fastest growing tumor (Fig.1, Table 3). Cell lines B2 and C4 did not induce tumors even after a nine-month follow up.

Histopathological analysis revealed that all the E$_2$ 70nM-C5 cells formed tumors and those tumors formed by their derived cells were poorly differentiated adenocarcinomas (Fig. 7). They invaded the mammary fat pad (Figs. 7a and 7b) and the skeletal muscle of the abdominal wall (Fig. 7c). Tumors formed by E$_2$ 70nM-C3 cells were also poorly differentiated adenocarcinomas; they were smaller and more circumscribed than E$_2$ 70nM-C5 formed tumors. BP1-Tras and MDA-MB231 cells also formed undifferentiated adenocarcinomas that were less invasive than those generated by E$_2$ 70nM-C5-derived cells.

The immunocytochemical reactivity of the E_2-induced tumors in SCID mice was compared with the reactivity of normal breast tissues, primary breast cancer, control MCF-10F cells, and with tumors formed by BP1-Tras and MDA-MB231 cells in SCID mice (Table 4). AE1 and AE3, human low and high molecular weight cytokeratins were expressed in the cytoplasm of the neoplastic cells in all E_2 induced tumors (Figs. 7e and 7g) in a pattern similar to those observed in normal breast tissues, in primary invasive ductal carcinomas of the breast (Figs. 7d and 7f) and in MCF-10Fcells (Table 4). The cytokeratin peptide 7 and 8 (CAM5.2) diffusely stained the cytoplasm of neoplastic cells with greater variations in the degree of intensity (Fig. 7i) than in the invasive ductal carcinoma of the breast used as positive control (Fig. 7h). E-cadherin was positive in all E_2-induced carcinomas, exhibiting a diffuse and moderate reactivity (Fig.7k), which was less intense than that observed in the invasive ductal carcinoma used as a positive control (Fig. 7j). Epithelial membrane antigen (EMA) had similar level of reactivity in E_2-induced tumors than in primary breast cancer, in normal breast tissues and in MCF-10F cells, but less intense that in tumors formed by BP1-Tras and MDA-MB231 in SCID mice (Table 4). Also the latter expressed high reactivity for vimentin (Table 4). Estrogen receptor alpha (ER-α), that was positive in normal breast tissues and in primary breast cancer was negative in MCF-10F cells and in all E_2-induced tumors in SCID mice. The same pattern of reactivity was observed for progesterone receptor (Table 4).

B-ii- c- *Fingerprint analysis*

MCF-10F cells transformed with 70nM E_2 and all the tumors and cells derived from them were used for fingerprint analysis that was performed using the six markers indicated in Table 1. All the tumors and cell lines derivate from MCF-10F showed the same sizes for the different markers (Table 2). The cell line MDA-MB-231 showed different sizes compared to MCF-10F and its derivates for five out of six markers tested (Table 2). These results indicated that all the tumors and cell lines tested, except MDA-MD231, originated from MCF-10F cells. These data were also confirmed using variable number of tandem repeat (VNTR) analysis. The Southern blot showed that the DNA profile of all of the cell lines have the same HinfI restriction pattern than MCF-10F cells, and different from MDA-MB-231 cells (data not shown).

B-ii- d- *Comparative genomic hybridization (CGH) analysis*

Estrogen treatment of MCF-10F cells resulted in losses and gains of genetic material that CGH showed to be progressive at the different stages of the tumorigenic process. In E_2-treated cells the first loss detected was in 9p11-13. The same loss was also maintained in 70nM-E_2-C5 cells, in the tumors formed by these cells in SCID mice and in all the cell lines derived from these tumors (Table 5). 70nM-E_2-C5 cells also exhibited loss of 4p, which expanded to the loss of the complete chromosome in the tumors derived from these cells as well as in the cell lines derived from the tumors (Table 5). Four additional losses appeared in all the tumors and in their derived cells that included 3p12.3-13, 8p11.1-21, 18q, and 9p21-pter, whereas the loss of 9p11-13 observed in previous cell lines was no longer evident (Table 5). Gains in 1p and 5q15-qter were observed in the four tumors formed by C5 cells in SCID mice (An1, An 4, An 6 and An 8) and the cell lines derived from them (C5-A1-T1, C5-A4-T4, C5-A6-T6 and C5-A8-T8) (Table 5). In the cell line 70 nM E_2-C5 that gave origin to the different tumors, the gain of 1p, 5q, and loss of chromosome 4 did not reach threshold values for being considered as gains or losses for xenografting, but in the tumors the C5 clone with these chromosomal alterations probably had a selective advantage and therefore these changes were very distinct.

Table 2: Fingerprint analysis

Cell lines	Markers					
	CSF1PO	TPOX	F13 AO1	VWA	FESFPS	THO1
MDA-MB-231	318/ 322	234/ 238	293/297	144/156	238	187/198
MCF-10F (P142)	310/318	238/246	293/297	144/152	238/246	191/198
BP1-Tras	310/318	238/246	293/297	144/152	238/246	191/198
E$_2$-70-MCF-10F (P20)	310/318	238/246	293/297		238/246	191/198
E$_2$-70-C3 (P16)	310/318	238/246	293/297	144/152	238/246	191/198
E$_2$-70-C5 (P16)	310/318	238/246	293/297	144/152	238/246	191/198
E$_2$-70-C5-An 1	310/318	238/246	293/297	144/152	238/246	191/198
E$_2$-70-C5-An 4	310/318	238/246	293/297	144/152	238/246	191/198
E$_2$-70-C5-An 6	310/318	238/246	293/297	144/152	238/246	191/198
E$_2$-70-C5-An 8	310/318	238/246	293/297	144/152	238/246	191/198
E$_2$-70-C5-A1-T1 (P3)	310/318	238/246	293/297	144/152	238/246	191/198
E$_2$-70-C5- A4-T4 (P3)	310/318	238/246	293/297	144/152	238/246	191/198
E$_2$-70-C5- A6-T6 (P3)	310/318	238/246	293/297	144/152	238/246	191/198
E$_2$-70-C5-A8-T8 (P3)	310/318	238/246	293/297	144/152	238/246	191/198

Table 3: Tumorigenesis induced by MCF-10F cells transformed with 17 β- estradiol

Group	N^{o}An.with tumors /N^o An. Injected	Mean Tumor size (mm) ±SD	Latency Period in days (From day of injection to appearance of first palpable mass [~2mm])
MCF-10F (p7-9 post-DMSO	0/10	0	NA
E$_2$-70 MCF-10F (p7-9)	0/10	0	NA
MDA-MB-231 (p13)	5/5	11.66±1.12	10-16
BP1-Tras	5/5	12.67±1.61	15-35
MCF-10F-A1	0/5	0	NA
MCF-10F-A2	0/5	0	NA
MCF-10F-A3	0/5	0	NA
MCF-10F-A4	0/5	0	NA
MCF-10F-A5	0/5	0	NA
MCF-10F-A6	0/5	0	NA
MCF-10F-A7	0/5	0	NA
E$_2$ 70-B2	0/3	0	NA
E$_2$ 70-C3	2/10	3.2±1.5	15-30
E$_2$ 70-C4	0/3	0	NA
E$_2$ 70-C5	9/10	10.92±2.73	5-30
E$_2$ 70-C5-A1-T1	5/5	11.80 ±3.67	10-15
E$_2$ 70-C5-A4-T 4	5/5	14.82±1.84	5-20
E$_2$ 70-C5-A6-T6	5/5	14.08±1.60	13-15
E$_2$ 70-C5-A8-T8	5/5	13.25±2.11	5-10

(i) Table 4: Histopathology and Immunocytochemical profile of 17 β-estradiol induced tumors

Group	Histopath ological type	AE1	AE3	CAM5.2	E-cadherin	EMA	Vimentin	ERα	
Normal breast	Normal breast	++	++	++	++	++	-	+	+
Breast cancer[1]	AdCa[2]	++	++	++	++	++	-	+	+
MCF-10F (p131)	Normal cells	+	+++	+	+	+	+	-	-
E$_2$-70-C5-A1	AdCa	+	+++	+	+	+	++	-	-
E$_2$-70-C5-A2	AdCa	+	+++	+	+	+	++	-	-
E$_2$-70-C5-A4	AdCa	+	+++	+	+	+	++	-	-
E$_2$-70-C5-A5	AdCa	+	+++	+	+	+	++	-	-
E$_2$-70-C5-A6	AdCa	+	+++	+	+	+	++	-	-
E$_2$-70-C5-A7	AdCa	+	+++	+	+	+	++	-	-
E$_2$-70-C5-A8	AdCa	+	+++	+	+	+	++	-	-
E$_2$-70-C5-A9	AdCa	+	+++	+	+	+	++	-	-
E$_2$-70-C5-A10	AdCa	+	+++	+	+	+	++	-	-
E$_2$-70-C5-A1-T1	AdCa	+	+++	+	+	+	++	-	-
E$_2$ 70-C5-A4-T4	AdCa	+	+++	+	+	+	++	-	-
E$_2$70nM-C5-A6-T6	AdCa	+	+++	+	+	+	++	-	-
E$_2$ 70nM-C5-A8-T8	AdCa	+	+++	+	+	+	++	-	-
BP1-Tras	AdCa	++	+++	+	+	+++	+++	-	-
MDA-MB-231 (p13)	AdCa	++	+++	++	+	+	+++	-	-

[1]Breast cancer, primary breast invasive ductal carcinoma; [2]AdCA, adenocarcinoma

B-iii *next proposed Plan of research*

B-iii-a-*To test if preventive agents are able to abrogate the transformation phenotypes.*
Tamoxifen will be used to block the action of estrogens through the receptor-mediated mechanisms. D3T, a monofunctional inducer of phase 2 enzymes, and NAcCys ± vitamins C and E will also be used as preventive compounds. The estrogen compounds will be used in the physiologic range of 0.007 nM to 70 nM, at which E$_2$ and metabolites induced transformation of HBEC. Dose-response curves for the preventive compounds will be established to determine the efficacious doses to be used. Basically the cells will be treated for two periods of 24 h each. The first treatment will be initiated 24 h post-plating of MCF-10F cells. The second treatment will be administered after 6 days. Aliquots will be collected after each treatment and after the cells have been selected in agar methocel. The data obtained will provide a direct evidence of the importance of affecting the homeostatic pathway of estrogen metabolism by measuring the transformation phenotypes in vitro and the tumorigenic response in the heterologous host.

B-iii-b-*Assays for anchorage-independent growth in agar methocel.* Treated and control cells collected will be seeded at a concentration of lxl0^4 cells in 0.8% methylcellulose in 24 multi-well plates pre-coated with a layer of 0.9% agar. The cells will be fed daily with fresh medium. All cultures will be evaluated 24 h post-plating for detection of cell aggregates that might bias the final results, and 21 days post-plating for determination of survival efficiency, colony number,

colony efficiency, and colony size.

B-iii-c-*Chemotaxis and invasiveness assays.* Chemotaxis and invasiveness will be determined using Boyden-type Transwell chambers (Costar, Cambridge, MA) separated by a porous polycarbonate filter (8 mm pore size) (Nucleopore, Pleasanton, CA) coated with reconstituted basement membrane material (Matrigel, Collaborative Research, Bedford, MA). Trypsinized cells will be seeded in the upper chamber and fibronectin at 1.0 µg/mL will be placed in the lower chamber as chemoattractant. The total number of cells that cross the membrane during a 12-h period of incubation will be determined under a light microscope upon fixation of the filters and staining by Diff Quick (Sigma Chemical Co., St. Louis, MO).

Table 5: Comparative Genomic Hybridization

Cell lines	Gains	Losses
MCF-10F		
E_2-70MCF-10F		9p11-13
E_2-70-C5		9p11-13; 4p
E_2-70-C5-A1	1p; 5q15-qter	3p12.3-13; 4; 8p11.1-21; 9p21-pter; 18q
E_2-70-C5-A4	1p; 5q15-qter	3p12.3-13; 4; 8p11.1-21; 9p21-pter; 18q
E_2-70-C5-A6	1p; 5q15-qter	3p12.3-13; 4; 8p11.1-21; 9p21-pter; 18q
E_2-70-C5-A8	1p; 5q15-qter	3p12.3-13; 4; 8p11.1-21; 9p21-pter; 18q
E_2-70-C5-A1-T1	1p; 5q15-qter	3p12.3-13; 4; 8p11.1-21; 9p21-pter; 18q
E_2-70-C5-A4-T4	1p; 5q15-qter	3p12.3-13; 4; 8p11.1-21; 9p21-pter; 18q
E_2-70-C5-A6-T6	1p; 5q15-qter	3p12.3-13; 4; 8p11.1-21; 9p21-pter; 18q
E_2-70-C5-A8-T8	1p; 5q15-qter	3p12.3-13; 4; 8p11.1-21; 9p21-pter; 18q

C. KEY RESEARCH ACCOMPLISHMENTS

1. *We demonstrate, to our knowledge for the first time, that 17β-estradiol induces complete in vitro transformation of human breast epithelial cells, as evidenced by the expression of anchorage independent growth, loss of ductulogenesis in collagen, invasiveness in Matrigel, and tumorigenesis in SCID mice*. Our previous work has demonstrated that treatment of the immortalized estrogen receptor alpha (ER-α) negative human breast epithelial cell line MCF-10F with E_2 and its metabolites 2- and 4-hydroxyestradiol induce anchorage independent growth, loss of ductulogenic pattern, and invasiveness in a Matrigel basement membrane (39-43,48). The transforming capabilities of estrogens have been confirmed in MCF-10A, another ER-α negative immortalized human breast epithelial cell line in which E_2 and estrogenic substances, such as Zeranol (Ralgro), a nonsteroidal agent with estrogenic activity that is used as a growth promoter in the U.S. beef and veal industry (49) and 4-hydroxyequilenin (50) induce anchorage

independent growth. These observations support the concept that estrogens induce neoplastic transformation through non-receptor alpha mediated mechanisms, exerting direct genotoxic effects, as previously suggested (32-38). Our findings of specific mutations in p53, and loss of heterozygosity (LOH) in chromosomes 11 and 13 further support this concept (40, 51).

2. *We report the novel finding that the tumorigenic phenotype is expressed only by E_2-transformed cells that after invading a Matrigel membrane exhibit a deletion of 4p15.3-16, a phenomenon that is preceded by the loss of 9p11-13, and followed by loss of the complete chromosome 4 during the tumorigenic process, accompanied by deletions in chromosomes 3p12.3-13, 8p11.1-21, 9p21-qter, and 18q, and gains in 1p, and 5q15-qter.* Using CGH that is a molecular cytogenetic method for screening gains and losses at chromosomal and subchromosomal levels, we have detected that MCF-10F cells transformed by E_2 had lost 9p11-13, a loss that persisted in the invasive cell line E_2-70 nM-C5. This locus contains the serine protease family member PRSS3 (trypsinogen-IV), a putative tumor suppressor gene (52) in which an allelic imbalance has been reported in hepatocellular carcinoma (53), carcinoma *in situ* of the bladder (54) and renal cell carcinoma (55). The loss of 9p11-13 was not detected by CGH technique in the tumors and tumor-derived cell lines, probably because the change did not reach the threshold for detection or because the cell population was heterogeneous. However, losses in 9p21-pter were clearly evident in the tumor and tumor cell lines. Losses of chromosome 9 regions are frequently reported in bladder carcinoma, especially in premalignant lesions such as hyperplasia and carcinoma in situ (CIS). Simultaneous losses in 9p11-q12 and in 9p21 have been reported in CIS of the bladder (56). Losses in this locus have also been reported in peripheral T cell lymphoma (57), melanoma cell lines (58), malignant fibrous histiocytoma (59) and parathyroid adenomas (60). The 9p21-pter, region includes both the p16 and p15 genes. These observations indicate that loss of these tumor suppressor genes on 9p contribute to the progression of the invasive to the tumorigenic phenotypes in the natural progression of the disease.

3. *We further demonstrate that E_2 induces, in addition to the expression of early phenotypes of neoplastic transformation, tumorigenesis in a heterologous host*. This phenomenon became possible only after the selection of invasive cells that exhibit specific changes, such as the deletion of chromosome 4p15.3-16, which was the first one, detected. Interestingly enough, injection of these cells to SCID mice resulted in the formation of tumors in which the entire chromosome 4 was deleted, a change that became a permanent feature of all tumors and tumor-derived cell lines. Allelic losses at one or both arms of chromosome 4 have been frequently reported in several tumor types, including breast cancers, either sporadic or occurring in BRCA1 and BRCA2 germline mutation carriers (61, 62). Regions that have been frequently reported to be deleted are 4p16.3 (50 %), 4p15.1-15.3 (57%), 4q25-26 (63%), and 4q33-34 (76%) (63).

4. *The tumors induced by E_2-transformed cells in SCID mice are fast growing and ER negative, being similar in these aspects to the tumors exhibiting similar deletions and that are diagnosed in young women, in whom tumors are large at the time of diagnosis, having a high percentage of cells in S-phase and being negative for estrogen receptors (61,62).* Chromosome 4 contains numerous genes of potential interest in cancer development, among them is *Slit2*, a gene located at 4p15.2 that encodes a protein that inhibits leukocyte chemotaxis and is a putative ligand for the ROBO receptors gene (64). *SLIT2* is primarily a secreted protein that in conditioned medium suppresses the growth of several breast cancer lines (64). Therefore the loss of the 4p15.3-16 region in E_2-70 nM C5 cells could be the event that triggers a cascade that

select tumorigenic cell population. Additional losses that were initially detected in the tumors and that were maintained in the tumor-derived cell lines were in chromosomes, 3 p12.3-13, 8 p11-21 and 18q. The region lost in chromosome 3 (p12.3-13) has been reported to exhibit imbalances in MCF-7 cells developing resistance to tamoxifen (65); the region 8p11-21 encodes the frizzled-related gene FRP1/FRZB, that is turned off in 78% of breast carcinomas (66), and associated with androgen in prostate cancer (67); the loss of chromosome arm 18q is a common event in primary breast cancers (68-72), ductal hyperplasia (73), and in breast cancer cell lines (74), and it is often interpreted as representing loss of one or more tumor-suppressor genes. The relevance of these losses in estrogen-induced cell transformation is that among the genes located in the q arm of chromosome 18 are two independent tumor-suppressor loci in segment 18q21.1, one at SMAD4 and the other potentially at an enhancer of DCC or an unrelated novel gene (68,72).

5. *Treatment of MCF-10F cells with E_2 induced genomic gains in 1p and 5q15-qter, both of which became evident in tumors and remained at the same level of expression in all tumor-derived cells*. Amplification of 1p has already been reported in primary breast cancer (75-80) and in established breast cancer cell lines (47). Gain in 5q15-qter has not been frequently found in breast cancer (81), but it has been reported in previously immortalized human ovarian surface epithelial (HOSE) cells using HPV16E6E7 ORFs (82) and in the cell lines SW480 and SW620, derived from different stages of colon carcinoma in the same patient (83). Although at the present time the role played by these gains in 1p and 5q15-qter in the process of estrogen-induced tumorigenesis is not known, a likely explanation is that the gains resulted from amplifications of smaller chromosomal segments that probably arose through real DNA amplification processes, suggesting that many genes present in these chromosomal loci are potential targets for the carcinogenic effect of 17-β-estradiol (84).

6. *Altogether our data indicate that 17-β-estradiol is able to induce complete neoplastic transformation of human breast epithelial cells, as proven by the formation of tumors in SCID mice.* This model demonstrates a sequence of chromosomal changes that correlates with specific stages of neoplastic progression. Our data also support the concept that 17-β-estradiol can act as a carcinogenic agent without the need of the ERα, although we cannot rule out thus far the possibility that other receptors such as ERβ, or other mechanisms could play a role in the transformation of human breast epithelial cells. These are areas of active research in our laboratory. The knowledge that breast cancer in women is associated with prolonged exposure to high levels of estrogens gives relevance to this model of estrogen induced carcinogenesis (6,8-10,15,16). For this reason this model is extremely valuable for furthering our understanding of estrogen induced carcinogenicity.

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E. CONCLUSIONS

During the third year of our award we have clearly demonstrated for the first time, that 17β-estradiol induces complete *in vitro* transformation of human breast epithelial cells, as evidenced by the expression of anchorage independent growth, loss of ductulogenesis in collagen,

invasiveness in Matrigel, and tumorigenesis in SCID mice. In this study we report the novel finding that the tumorigenic phenotype is expressed only by E_2-transformed cells that after invading a Matrigel membrane exhibit a deletion of 4p15.3-16, a phenomenon that is preceded by the loss of 9p11-13, and followed by loss of the complete chromosome 4 during the tumorigenic process, accompanied by deletions in chromosomes 3p12.3-13, 8p11.1-21, 9p21-qter, and 18q, and gains in 1p, and 5q15-qter.

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April 18, 2006

Commander
U.S. Army Medical Research and Materiel Command
ATTN: MCMR-PLF (BCRP BAA 96)
Building 1076
Fort Detrick
Frederick, Maryland 21702-5024

Dear Sir:
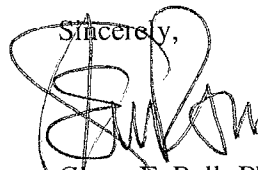
The protocol(s) pertaining to this application submitted to the U.S. Army Medical Research and Materiel Command was reviewed and approved by this Institution's Animal Care and Use Committee on January 25, 2006.

Title of Application: Estrogen Induced Depurination of DNA: A Novel Pathway of Breast Cancer Prevention

Name of Principal Investigator: Dr. Jose Russo

Name of Instituion: Fox Chase Cancer Center

This Institution has an Animal Welfare Assurance on file with Office of Laboratory Animal Welfare. The Assurance number is A3285-01.

Sincerely,

Glenn F. Rall, Ph.D.
Chairman
Institutional Animal Care and Use Committee

GFR/atp

SPECIFIC AIM 3 - GUTTENPLAN

Introduction

Oxidation of 2-OHE$_1$/E$_2$ results in the formation of E$_1$/E$_2$-2,3-Q, which forms primarily stable adducts in DNA (1). On the other hand, oxidation of 4-OHE$_1$/E$_2$ results in the formation of E$_1$/E$_2$-3,4-Q, which forms primarily depurinating DNA adducts (1). Since the quinone (E$_1$/E$_2$-3,4-Q) of the more potently carcinogenic 4-OHE$_1$/E$_2$ forms primarily depurinating adducts, we hypothesize that estrogen-induced depurinating adducts have a central role in the initiation of breast cancer. As depurinating adducts leave DNA, apurinic sites are left behind. Recently, it was found that apurinic sites formed by E$_2$-3,4-Q in mouse skin induced mutations by error-prone DNA repair (2). This suggests that estrogen may induce mutations by apurinic site misrepair in certain cancer genes to initiate sporadic breast cancer. Our hypothesis in this specific aim is that in a *lacI* rat model for *in vivo* mutagenesis, estradiol will be metabolized to 4-OH-estradiol (4-OHE$_2$), and 4-OHE$_2$ can then be converted to estradiol-3,4-quinone (E$_2$-3,4-Q). This latter compound will be mutagenic.

In the previous year we found that extended exposure (20 weeks) of BigBlue Fisher 344 rats to 4-OHE$_2$, E$_2$, or a combination of the two agents led to a modest increase in the mutant fraction in mammary tissue from the treated rats. The mutant fraction was approximately doubled compared to background in all 3 groups. The mutational specificity was determined and the groups that received 4-OHE$_2$ contained about twice the fraction of mutations at AT base pairs as the other groups. This specificity is consistent with the observation that a significant fraction of DNA adducts produced by E$_2$-3,4-Q is found at adenines (particularly the N-3 position) (3). Similarly, in cultured BigBlue rat embryonic fibroblasts, we found 4-OHE$_2$ led to a modest increase in the mutant fraction when compared with the background in untreated cells, and the mutational spectrum of the 4-OHE$_2$-induced mutations was similar to that observed in the rats (4).

One major goal of this project is to identify potential inhibitors of DNA damage by estrogen metabolites. The mutagenesis assays are, in principle, valuable screening assays for chemopreventive agents, as they require few rats and are relatively short when conducted *in vivo*. The *in vitro* cell culture assays are much simpler, but the results must then be validated in an *in vivo* system. One problem with the *in vivo* and *in vitro* mutagenesis assays is that the 4-OHE$_2$-induced mutagenesis produces less than a doubling in the mutant fraction. As a result, only extremely effective inhibitors would produce statistically significant inhibition. Therefore, modest inhibitors might be missed. To overcome this problem, in the past year, we have been investigating methods to increase the mutant fraction induced by estrogen and its metabolites.

Methods

Newborn rats

We investigated the use of newborn rats (7 – 11/group), treated for five days ip. with 4-OHE$_2$ and E$_2$- 3,4-Q (the putative ultimate mutagen and carcinogen). Newborn animals are often more sensitive to carcinogens than adults. The rats were then kept for 20 weeks and the mutagenesis in mammary tissue and liver was measured and other organs were kept frozen for possible future studies. For mammary tissue, the mean of the mutant fraction in a row of four

thoracic glands from each rat was determined. Mammary tissue, uterus, ovaries and liver were isolated and frozen.

Long-term slow release study: high dose
        Our previous long-term model was carried out using 5 mg/rat of 4-OHE$_2$, E$_2$, or a combination the two agents in a silastic tubing intra-scapular implant. In the current study we then attempted to increase the mutant fraction by increasing the dose to 15 mg/rat. We also tested 5 and 15 mg of 2-OHE$_2$, which should be a weaker mutagen, according to our hypothesis. In addition, we tested diethylstilbestrol (DES), and 3-OH-DES, a metabolite of DES. Finally, E$_2$ and a control group were also treated. Mammary tissue, uterus, ovaries and liver were isolated and frozen. Groups of 6-8 rats were treated for 20 weeks.

Polymerase-β-deficient (polβ) *lacI* mouse cell line
        In the previous year, we also determined that 4-OHE$_2$, but not 2-OHE$_2$, was mutagenic in a rat embryonic *lacI* cell line. The potency was low and multiple exposures were necessary to observe statistically significant increases in the mutant fraction (MF). We have recently obtained a polymerase-β-deficient *lacI* mouse cell line. As polymerase-β is necessary for repair of gaps produced in the excision of many DNA adducts, we hypothesized that its deficiency would increase the sensitivity of the cells to genotoxic damage induced by estrogens.

Results

Newborn study
        Mutagenesis in mammary tissue and liver was measured. Although there was a small increase in the mutant fraction, particularly for the quinone, the increase was not statistically significant and was less than that seen in previous long-term exposure studies. It was concluded that in mammary tissue this model was less sensitive than the previous long-term exposure model, (perhaps because of the limited exposure time) and therefore would not be suitable for studies on inhibition. We also measured mutagenesis in the liver and also observed a modest increase in mutagenesis in the treated groups. This increase failed to reach statistical significance. The results of this study are given in Table 1. Other organs from these animals have been kept in storage at -80°C and may be useful in future studies (see below).

Table 1. Effect of Estrogens on Mutagenesis in Newborn *lac*I rats

		mammary tissue		liver	
compound	n	MF[1]	SD	MF	SD
control	8	0.80	0.22	0.69	0.29
4-OHE$_2$	7	0.85	0.46	0.94	0.58
E$_2$-3,4 quinone	11	1.09	0.40	0.90	0.34

[1]Mutant fraction x 10^5 pfu

Long-term slow release study
        We are still analyzing some of the tissues from this group, but analysis of several groups is near completion. The results obtained thus far are given in Table 2. A modest increase in mutagenesis was observed in the 5 and 15 mg 4-OHE$_2$-treated groups and in the 3-OH-DES group, although the increase was only statistically significant in the 3-OH-DES group. However,

not all glands in all of the animals have been analyzed and it seems likely that with more data points the variance will decrease and significance in the 4-OHE$_2$ groups may be obtained. No increase in mutagenesis was seen in the one 2-OHE$_2$ group thus far tested.

Although the MF in both 4-OHE$_2$ groups was increased, the values in these groups were essentially identical. The reason for the lack of dose-response in mutagenesis by 4-OHE$_2$ may lie in the physical characteristics of release of the estrogens from the silastic tubing. At a certain point, the permeability is no longer limited by the concentration of the solute, but rather, the surface area of the tubing.

Table 2. Effect of Estrogens on Mutagenesis in Mammary Tissue in lacI rats

compound	n	MF[1]	SD
control	5	0.81	0.51
2-OHE$_2$ (5 mg)	7	0.87	0.61
4-OHE$_2$ (5 mg)	8	1.29	0.41
4-OHE$_2$ (15 mg)	6	1.26	0.15
3-OH-DES (5 mg)*	6	1.67	0.30

[1]Mutant fraction x 10^5 pfu
*p = 0.01 vs control

Pol-β cell line

We have started characterizing this cell line with respect to its sensitivity towards standard mutagens and its background. The background is 8-9 x 10^{-5} mutants/pfu. This is about twice that of the *lacI* rat cell line previously employed. We cannot yet determine whether this results from the different DNA repair of endogenous lesions or is simply a characteristic of the parent repair proficient cell line. We have also found that the polymerase-β cell line is about twice as sensitive to the mutagenic effects of benzo[*a*]pyrene than the rat embryonic *lacI* cell line.

Catechol-O-methyltransferase (COMT) inhibitors

According to our hypothesis, the conversion of 4-OHE$_2$ to E$_2$-3,4-Q can be partially prevented by its conversion to its O-methoxy derivative. This would decrease the concentration of 4-OHE$_2$ available for conversion to the quinone. In order to inhibit this conversion in cell culture we have employed the COMT inhibitor, RO41. We treated the l*ac*I cell with 4-OHE$_2$ with and without inhibitor, and had media analyzed by Dr. Zahid (U. Nebraska Medical Center) for the presence of depurinated guanine and adenine adducts, that should be produced from the reaction of the E$_2$-3,4-Q with DNA. If the inhibitor prevented conversion of 4-OHE$_2$ to the methoxy derivative, we should detect increased levels of DNA adducts in the media. This was indeed the case. The concentration of the 4-OHE$_2$-1-N3Ade and 4-OHE$_2$-1-N7Gua adducts increased from about 1 pmol/10^6 cells to about 2.2 pmol/10^6 cells in the presence of the COMT inhibitor.

Proposed Research for the Coming Year

Since we were thus far not able to increase the sensitivity of the mutagenesis assay for 4-OHE$_2$ in mammary tissue, we are considering the possibility of finding a surrogate tissue. For

instance, it is known that the hamster kidney and mouse uterus represent models for cancer induction by estrogens (5). As indicated above, we have saved several organs from the newborn and long-term exposure groups. We plan to assay mutagenesis in uterus and ovary of these groups and the other groups (2-OHE$_2$, 3-OH-DES, and DES). In addition, mutagenesis induced by these other groups of estrogens in *lac*I rat mammary tissue will be analyzed.

We also plan to continue sequencing mutants from estrogen-treated rats and determine differences in the mutational spectra between different compounds.

We are currently planning to test the possibility that the reason for the lack of a dose-response in mutagenesis induced by 4-OHE$_2$ in *lacI* rats is a plateau in the rate of release of 4-OHE$_2$ from the silastic tubing (see above). This possibility will be investigated by measuring blood levels of 4-OHE$_2$ after implanting different concentrations in silastic tubing. If blood levels do not increase with increasing levels of 4-OHE$_2$ in the implant, then permeation of estrogen from the tubing must be a limiting factor. If this occurs, then other means of exposure, such as minipumps or pellets will be investigated.

We also plan to test the polβ cell line as a more sensitive target for mutagenesis induced by estrogen metabolites. The first exposures using this cell line have recently begun and we are collecting cells after 1, 3 and 6 treatments with 4-OHE$_2$, with and without COMT inhibitor. We are also treating with the E$_2$-3,4 and 2,3 quinones to determine whether mutagenesis induced by the hypothesized ultimate mutagens can be observed in this cell line.

Key Research Accomplishments

1.   Demonstrating that 4-OHE$_2$ was more mutagenic than 2-OHE$_2$ in *lac*I rat mammary tissue.
2.   Demonstrating that 3-OH-DES is mutagenic in *lac*I mammary tissue.
3.   Demonstrating that a COMT inhibitor increased the level of depurinating adducts in *lacI* cells treated with 4-OHE$_2$.
4.   Validating the *lacI* polβ cell line.

Reportable outcomes

1.   4-OHE$_2$ is mutagenic in *lac*I rat mammary tissue.
2.   4-OHE$_2$ was more mutagenic than 2-OHE$_2$ in *lac*I rat mammary tissue.
3.   3-OH-DES is mutagenic in *lac*I mammary tissue.
Note: The experiments demonstrating the above outcomes are not yet complete. More quantitative data will be obtained before publishing the results.

Conclusions

Estrogen and DES metabolites are mutagenic in *lac*I rats, but the increase in mutant fraction (above background) is small. We have not yet found a more sensitive assay for mutagenesis induced by estrogen metabolites, and we are exploring other exposure methods or the use of a surrogate organ (uterus, or ovary).

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SPECIFIC AIM 4 - SANTEN

Specific Aim 4-a. Depurinating CE-DNA metabolites in breast tissue of aromatase transfected mice.
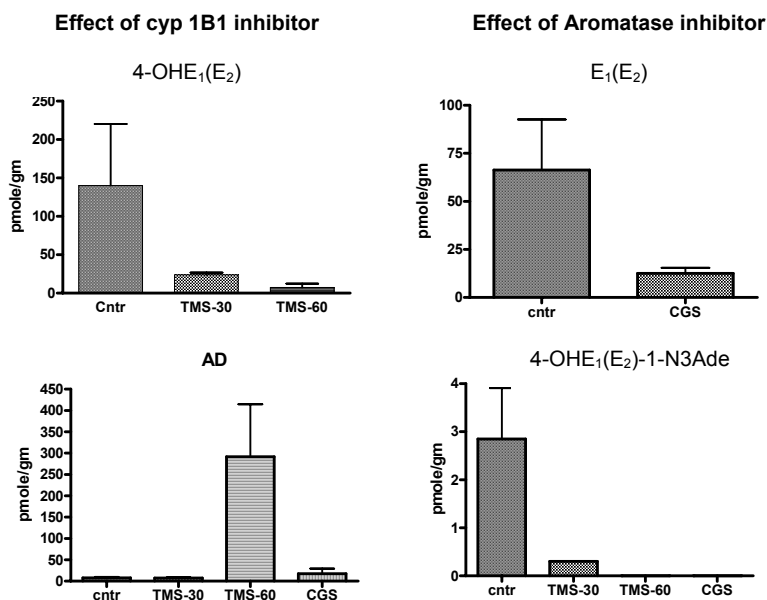

Figure 1

    Aromatase transgenic mice are engineered to over-express the aromatase gene specifically in breast tissue. These animals, provided by Dr. Raj Tekmal, were used to determine the basal levels of catechol estrogen metabolites in untreated animals and the levels after treatment with TMS (tetramethoxystilbene), an inhibitor of cytochrome p450 1B1 (i.e. blockade of 4-hydroxylation of estradiol) and letrozole, an inhibitor of aromatase. Figure 1 above demonstrates that the basal levels of 4-OH estrone and estradiol [4-OH-$E_1(E_2)$] were 140 pmole/gm and fell to 25 pmol/gm with 30 mg/kg of TMS and to 10 pmol/gm with 60 mg/kg TMS. This establishes that TMS is a potent inhibitor of 1B1 in vivo and confirms our results showing blockade of this enzyme in vitro (Data not shown).

    The goal of blocking 1B1 is to reduce the amount of depurinating adduct present in the breast tissue. Under control conditions, we detected 2.8 pmole/gm of 4-OHE$_1$(E$_2$)-1-N3Ade. In response to 30 mg/kg TMS, levels were reduced to 0.25 and with 60 mg/kg TMS, the amount was undetectable. The estradiol-adenine adduct results when the estradiol –quinone binds covalently to adenine and then causes non-enzymatic cleavage to the free 4-OHE$_1$(E$_2$)-1-N3Ade adduct. The presence of this product is a direct demonstration of the ability of estradiol to be metabolized to genotoxic compounds which can bind to adenine and cause depurination. This experiment also demonstrated that TMS can block 1B1 and formation of depurinated product in a dose dependent fashion *in vivo* in aromatase transgenic animals.

Not shown in the figure is that under control conditions, the breast tissue of these animals also contains measurable amounts of the other depurinated adduct, 4-OHE$_1$(E$_2$)-1-N7Gua, as well as several other estradiol metabolites including 4-methoxy-E$_1$(E$_2$), the glutathione conjugate of 4-OHE$_1$(E$_2$) which is designated 4-OHE$_1$(E$_2$)-2-SG, the *N*-acetylcysteine conjugate termed 4-OHE$_1$(E$_2$)-2 NAcCys and the cysteine conjugate called 4-OHE$_1$(E$_2$) Cys. All of these compounds were suppressed by the 60 mg/kg dose of TMS.

The aromatase inhibitor letrozole was also given to the aromatase transfected animals and breast metabolite levels measured. As shown in Figure 1, above, the levels of E$_2$ and E$_1$ were reduced from 65 pmole/gm under basal conditions to 10 pmole/gm during treatment with letrozole (CGS). This verified the efficacy of letrozole in blocking tissue levels of free estrogens. In response to this, the level of the 4-OHE$_1$(E$_2$)-1-N3Ade depurinating adduct became undetectable and its N7Gua counterpart was suppressed as well.

Taken together, these experiments demonstrate both the presence of depurinating adducts but also the ability to suppress these with a 1B1 inhibitor as well as an aromatase inhibitor.

Biologic studies with TMS were then conducted in cell lines and in xenografts. *In vitro* studies utilized three cell lines: (1) ER (+) MCF-7 breast cancer cells; (2) long-term tamoxifen treated MCF-7 cells, which were characterized by increased cross-talk between ERα and EGFR; and (3) long-term fulvestrant treated MCF-7 cells with up-regulated EGFR and HER2. *In vivo* studies used a tamoxifen-resistant MCF-7 breast cancer xenograft model. TMS caused E$_2$ independent inhibition of the proliferation of MCF-7 cells. Using an ELISA apoptosis kit, we demonstrated a dose-dependent increase in apoptosis with 0.03 µM to 9 µM TMS given in conjunction with E$_2$. During examination of the mechanisms involved, we showed that TMS inhibited phosphorylation of MAPK (both ERK 1 and 2), focal adhesion kinase (FAK), Akt, and mammalian target of rapamycin (mTOR) and stimulated c-Jun N-terminal kinase (JNK) and p38 MAPK activity. A dose of 0.3 µM TMS showed 80% growth inhibition of long-term tamoxifen treated MCF-7 cells and 70% growth inhibition of fulvestrant treated MCF-7 cells. *In vivo* studies (8 weeks treatment with TMS via a 30 mg subcutaneous implant) reduced tumor volume of estrogen-independent and tamoxifen resistant MCF-7 breast cancer xenografts by 53%.

We then examined the effect of TMS on the aromatase transgenic animals. The 30 and 60 mg/kg doses caused a marked reduction of the development of ductal, lobular and alveolar structures in 4 month old mice. This effect could not be overcome by the administration of E$_2$ sufficient to produce blood levels of 150 pg/ml. In order to quantitate the morphologic changes, we utilized a computer program which calculates the percent area occupied by ductal, lobular and alveolar (DLA) elements compared to total breast area in whole mounts of breast. These measurements demonstrated a 75% reduction in DLA with the 60 mg/kg dose, an effect statistically significant at the $p<0.001$ level. When the TMS was withdrawn and DLA examined one month later, the decrements were completely reversible. Ongoing experiments are assessing the ability of 4-OHE$_2$ to rescue these effects.

We have assessed the toxicity of TMS in these animals in several ways. No change in total body, uterine, or ovarian weight was observed after one month of TMS treatment at the 60 mg/kg dose. Measurement of E$_2$ by radioimmunoassay also detected no change in plasma E$_2$. Histologic examination of liver, kidney, and ovary also detected no apparent changes. These data strongly suggest the intriguing finding that biologic effects of TMS are specific to breast

and that this compound does not exhibit systemic toxicity. Further studies are planned to explore why TMS is specific to breast. Taken together, these results suggest that TMS could be effective in preventing breast cancer in experimental models and potentially in women.

Specific Aim 4b. Serial transplants. The aromatase transfected mammary glands have now been serially transplanted into additional animals according to the method of Daniel Medina. We have not yet observed tumor development. These experiments are ongoing as described in the original grant proposal. We expect that after another 12 months we may begin observing tumor development in these mammary glands.

Specific Aim 4c. Development of breast cancer in the absence of a functional estrogen receptor. *Morphology of the mammary gland* Mammary glands from wild type (ER+) mice contain ductal and lobulo-alveolar structures which fill the entire mammary fat pad at 6 months of age, whereas knock-out of ERα results in only rudimentary mammary ductal structures. Introduction of the Wnt-1 gene into ERKO animals caused proliferation of the existing mammary rudiment, but these structures still occupied only a small portion of the mammary fat pad. In the ERKO animals, the Wnt-1 gene, rather than E_2, appeared to be the primary mediator of ductal and lobular-alveolar development. As a result, neither oophorectomy (OVX) before day 16 of age, oophorectomy plus 240 pg/ml E_2 for at least two months (Ovx+E_2), nor oophorectomy plus E_2 plus fulvestrant (Ovx+E_2+ICI) substantially altered the mammary morphology of the ERKO/Wnt-1 animals.

In the intact animals containing wild type ER and Wnt-1, the Wnt-1 gene resulted in exuberant growth of ducts and lobulo-alveolar structures and mammary structures filled the entire mammary fat pad. In these ER+ wild type/Wnt-1 animals, E_2 continued to influence mammary growth since oophorectomy reduced the degree of ductal and alveolar development (Ovx), an effect overcome by exogenous E_2 administration (Ovx+E_2). Fulvestrant completely blocked this effect of E_2 since the mammary tissues in the oophorectomized and fulvestrant plus E_2 treated animals (Ovx+E_2+ICI) appeared to be similar to the appearance of the castrate (Ovx) animals. Taken together, these results confirm the unresponsiveness of the ERKO/Wnt-1 mammary glands to E_2 resistance to E_2 and the maintenance of an ER mediated effect in the ER+/Wnt-1 glands.

Presence of estradiol metabolites in breast tumor tissue
With development of the mass spectrometry method to confirm metabolite formation in tissues, we examined four breast tumors developing in ERKO animals. After pooling of two tumors each to enhance tissue mass, we measured multiple metabolites with the mass spectrometry method. As evidence of the formation of depurinated products, we detected the presence of 4-OHE$_2$-1-N3Ade at levels of 0.03 and 0.49 pmol/gm and 4-OHE$_1$-N3Ade concentrations of 0.19 and 0.29 pmol/gm respectively. With respect to the 4-OHE$_1$-1-N7Gua, we detected 0.21 and 0.35 pmol/gm but did not detect the E_2 counterpart. As confirmation of previous studies, the tumors contained higher amounts of the 4-hydroxylated free estrogens, the methoxyestrogens, and the depurinated products than the respective 2-hydroxylated compounds. This reflects the metabolic imbalance with formation of the more genotoxic 4-hydroxylated metabolites. In contrast to prior findings, we did detect significant amounts of the methoxy-derivatives, suggesting the presence of catechol-*O*-methyltransferase (COMT) in these tumors. For example, we detected 14.6 and 16.8 pmol/gm of the 4-OCH$_3$E$_2$ in the tumors. Taken

together, these experiments document the presence of the enzymes necessary to metabolize estrogens to the genotoxic quinones and the formation of depurinating DNA adducts.

Effect of ovarian hormones in the absence of ERα

Removal of the ovaries before day 16 in the ERKO animals allowed examination of the role of ovarian factors acting in the absence of ERα. This ablative procedure delayed the 50% incidence time point to 24 months and reduced the number of animals with tumors to 50% relative to intact ERKO mice. It should be noted that the ovary produces other steroid and peptide hormones, such as progesterone, that can influence mammary growth. In order to examine the role of E_2 in the absence of ovarian factors, we treated castrate ERKO animals with E_2 over a 24 month period and maintained E_2 levels at 5, 10, 80 and 240 pg/ml with the "estradiol clamp" method. The 240 pg/ml dose in the castrate animals caused tumors to develop earlier, with a 50% incidence time point at 10 months versus 24 months in vehicle-treated animals. As in the intact animals, nearly 100% of E_2 treated animals developed tumors. The 80 pg/ml E_2 dose produced effects intermediate between those of vehicle and 240 pg/ml E_2. The 5 and 10 pg/ml E_2 doses appeared to be sub-threshold with no differences compared to vehicle.

Complete elimination of effects of truncated ERα and ERβ

Prior studies had shown that the ERKO mice utilized in this study expressed an mRNA species that if translated would yield a 56 Kd truncated ERα, which retained the DNA and ligand binding domains. In the ERKO animals, ER binding activity was reduced by 91-99% in uterine cytosols and E_2 responsiveness by 80-90%. ERβ, while not found on RNase protection assays, could be detected by the more sensitive quantitative PCR methodology. Recognizing the need to eliminate the biologic effects of these possible residual receptors, we completely abrogated ERα and β function with fulvestrant and examined the effect of E_2 under these conditions. Fulvestrant or vehicle was administered to castrate ERKO animals with E_2 clamped at 240 pg/ml. The tumors appeared at the same rate in the presence or absence of fulvestrant, providing evidence that the effect of E_2 was not mediated by a truncated ERα or low level ERβ. A further strategy was also chosen to eliminate receptor mediated effects by the administration of 17α-OHE$_2$. This compound lacks ER mediated effects on uterine weight, but is capable of forming potentially genotoxic compounds. 17α–OHE$_2$ induced tumors in the ERKO animals at a rate identical to that in animals with E_2 maintained at the same plasma level (i.e. 240 pg/ml).

Bioassay of "clamped" E_2 on uterine weight

Our various strategies to examine the ER-independent effects of E_2 critically depended upon complete blockade of any residual ER activity resulting from a truncated ERα or low level ERβ. Measurement of uterine weight provided a robust bioassay of the tissue effects of E_2 to determine if complete blockade was achieved. We measured uterine weight after at least two months of E_2 exposure under each experimental condition. Uterine weights in the intact (i.e. non-castrate) ER+ animals were 84 ± 12 mg (mean ± SE) and in the ER-, 28 ± 3 mg. Ovariectomy (ovx) reduced uterine weights to 5 and 4 mg, respectively, in both the ER+ and ER- animals. This observation suggested that the ERKO animals were still capable of responding to endogenous estrogen (i.e. difference between intact and castrate ERKO). Administration of E_2 by the "clamp" method increased uterine weight to 164 ± 5 mg in the ER+ animals and to 24 ± 3 mg in the ER-. This blunted increase in uterine weight in ERKO animals represented only 18% responsiveness and possibly represented an effect of the truncated ERα receptor. The administration of fulvestrant completely blocked the residual ER responsiveness in the ERKO animals since uterine weight fell to 7 ± 1 mg in the animals receiving 240 pg/ml E_2. Fulvestrant

also blocked uterine weight in the E_2 treated ER+ animals by approximately the same percentage (i.e. 164 to 22 mg or 83% reduction) as in ERKO animals but the absolute reduction in uterine weight was less (22 mg). In aggregate, these data demonstrated that fulvestrant was capable of completely abrogating the effects of residual ER activity in ERKO animals.

We wished to confirm by bioassay that 17α-OHE$_2$ also did not stimulate the uterus. At a level of 240 pg/ml this compound caused no increase in uterine weight (4 ± 0.5 mg) indicating its lack of uterotrophic activity. As further proof of the minimal ER mediated effects of this compound, we tested its ability to stimulate transcription of endogenous and exogenous estrogen responsive genes and MCF-7 cell growth *in vitro*. The potency of 17α-OHE$_2$ on progesterone receptor synthesis (endogenous genes), on transcription of ERE-luciferase construct (exogenous reporter gene), and on cell growth was 1% that of E_2 itself.

Taken together, these results provide convincing evidence that E_2 can influence breast tumor formation in the absence of functioning estrogen receptors. These results and the demonstration that the breast tissue of ERKO animals can metabolize E_2 to genotoxic metabolites provide strong support for the depurination/genotoxic estrogen metabolite hypothesis to explain the development of breast tumors.

Publications and Abstracts

1.   Publication Submitted to PNAS June 5, 2006**:** Estrogen effects on development of breast cancer: involvement of both ER dependent and independent mechanisms

   Wei Yue, Ji-Ping Wang, Yuebai Li, Ping Fan, Giujian Liu, Nan Zhang, Ken Korach, Wayne Bocchinfuso, and Richard Santen

2.   Abstract of Oral Presentation: 88th Annual Meeting of the Endocrine Society, Boston, Massachusetts, June 23-27, 2006 Treatment of hormone resistant breast cancer with new inhibitor of catecholestrogens: Beyond chemotherapy

   Ho-Yong Park, Ping Fan, Jiping Wang, Wei Yue, Robert X. Song, Sang-Hee Kim, Richard J Santen

3.   Abstract submitted to the San Antonio Breast Cancer Symposium for December 15, 2006: Could inhibitor of catecholestrogens be an ideal agent to prevent breast cancer in premenopausal women?

   H Y Park, EG Rogan, EL Cavalieri , P Fan[3], J P Wang, W Yue, S M Hong, K Mohammad, R R Tekmal, M A Jordan, L M Demers, V Chandrashekar, S H Kim, Y H Lee and R J Santen.

4.   Abstract presented at the American Association of Cancer Research ,Washington DC, March 2006: Prevention of breast cancer and Treatment of hormone resistance breast cancer with new inhibitor of catecholestrogens

   Ho-Yong Park, Ping Fan, Ji-Ping Wang, Wei Yue, Robert X. Song, Seung-Mo Hong, Sang-Hee Kim, Rajeshwar Rao Tekmal, Richard J Santen[2]

SPECIFIC AIM 5 – INGLE

A.   Introduction
        Breast cancer remains a major burden for many women around the world and is a concern for most women. Much of the clinical research to date has involved large and hugely expensive clinical trials. The most recently reported is that of the Study of Tamoxifen and Raloxifene (STAR) that involved over 19,000 women accrued over six years with a reported cost of about $118,000,000. The current major efforts in prevention involve placebo-controlled trials of exemestane (MAP.3) involving over 5,000 patients, and anastrazole (IBIS 2) that involves 6,000 women. The next planned large trials will involve a comparison of raloxifene versus an aromatase inhibitor. It is clear that new approaches and paradigms are needed to identify biologically-based strategies for prevention of breast cancer. It would of great value to identify biomarkers that would allow targeted studies involving smaller sample sizes at much lower cost.

B.   Body

1.   Methods
        The approach taken in Specific Aim 5 has been to develop and conduct a prospective study aimed at determining levels of estrogen, catechol estrogen metabolites, catechol estrogen-glutathione conjugates, and most importantly, catechol estrogen-DNA adducts in women at high risk of developing breast cancer. This protocol was developed at Mayo Clinic and is entitled, "Evaluation of the Estrogen Profile in Nipple Aspirate Fluid in Women with a Personal History of Breast Cancer and those at High Risk" (IRB# 901-05). The protocol was amended in March 2006 to also obtain urine and blood because of the findings by Drs. Cavalieri and Rogan that DNA adducts were able to be identified in these samples. This protocol has been activated and submitted for funding. To date the conduct of this study has been supported by Dr. Ingle as head of the Mayo Breast Cancer Research Program. The protocol is appended and the goal is to obtain specimens on 160 women over a two year period of time.

2.   Results
        Specimen collection is ongoing.

3.   Proposed plan of Research for the Next Year
        The major goal will be to complete collection of the specimens as indicated in the protocol discussed above.

C.   Key Research Accomplishments
        As yet, the major accomplishment has been the development and activation of the protocol, but no specific research accomplishments are available as of yet.

D.   Reportable Outcomes
        None.

E.   Conclusions
        The levels of the estrogens, estrogen metabolites, and catechol estrogen-DNA adducts represent promising biomarkers for use in future prevention studies.

F.   References
        None.

Mayo Clinic Cancer Center

Estrogen-DNA Adducts in Breast, Urine and Serum as Biomarkers of Breast Cancer Risk

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Document History	Implementation Date
Activation	May 20, 2005
Mayo Addendum 1	March 7, 2006
Mayo Addendum 2	March 22, 2006
Mayo Addendum 3	June 20, 2006

1. **Protocol Title:**
   Estrogen-DNA Adducts in Breast, Urine and Serum as Biomarkers of Breast Cancer Risk

2. **Phase:** Not applicable.

3. **Principal Investigator - Sandhya Pruthi, MD**
      Co- Investigator - James L. Ingle, MD
      Mayo Clinic, Rochester, MN
      Mayo Breast Diagnostic Clinic

   Collaboration and Investigators: Ercole Cavalieri, D.Sc., Professor, Eppley Institute for Research in Cancer, 986805 Nebraska Medical Center, Omaha, NE 68198-6805

   Eleanor Rogan, Ph.D., Professor, Eppley Institute for Research in Cancer, 986805 Nebraska Medical Center, Omaha, NE 68198-6805

4. **Location of Study:** Mayo Clinic, Rochester, MN

5. **Time Required to Complete:** 4/1/2005 – 4/2007 Two years

6. **Objectives:** We propose that a major carcinogenic risk for human breast cancer is associated with endogenous catechol estrogens (CE, both 2-CE and 4-CE), which can be activated to ultimate carcinogenic forms, namely quinones (CE-Q) [1,2]. Estrogens are oxidized to 4-CE primarily by cytochrome P450 (CYP) 1B1 [3]. An efficient protective mechanism that impedes oxidation of 4-CE to CE-3,4-Q is monomethylation at the 2-, 3- or 4-hydroxyl group catalyzed by catechol-O-methyltransferase (COMT). With elevated rates of CE synthesis and/or deficient methylation, significant quantities of 4-CE are available for oxidation to CE-3, 4-Q by peroxidases or cytochrome P-450. Covalent binding of CE-3, 4-Q to DNA leads to DNA damage that could initiate the cancer process [1, 2]. This damage is the formation of the depurinating DNA adducts, 4-hydroxyestradiol (estrone)-1-N7guanine and 4-hydroxyestradiol (estrone)-1-N3adenine. Such DNA adducts are released from DNA by breaking the bond between the purine base (adenine or guanine) and the deoxyribose, a process called "depurination". The resulting "apurinic sites" in the DNA, gaps with no base, can cause mutations to occur at these sites. CE-Q also react with glutathione (GSH) to form conjugates that are present in tissues and excreted in urine as CE-GSH, CE-cysteine (Cys) and CE-*N*-acetylCys (CE-NAcCys) conjugates [4-6].

   With the purpose of supporting our hypothesis and developing bioassays for susceptibility to breast cancer, we plan to conduct a preliminary study in humans to determine the levels of CE, CE metabolites, CE-DNA adducts and CE conjugates in nipple aspirate fluid samples obtained from women at elevated risk for the development of breast cancer and women with breast cancer. CE, conjugates and adducts will be identified and quantified in the breast fluid by HPLC with electrochemical/mass spectrometric detection.

   The analyses of CE, conjugates and DNA adducts will be conducted under the supervision of Dr. Eleanor Rogan at UNMC, in a manner similar to the analyses of CE and CE conjugates

in breast tissue conducted previously [7]. Nipple aspirate fluid has been used in epidemiological studies by analyzing various biochemicals, including lactose, cholesterol, estrogens, androgens and related compounds for almost 20 years [8-10]. Recently Chatterton, et al., analyzed the levels of estradiol, estrone and estrone sulfate with improved sensitivity [11].

Drs. Cavalieri and Rogan's laboratory recently analyzed three additional nipple aspirate samples from healthy control women who have not been diagnosed with breast cancer. As seen in Figure 1, the 2-catechol estrogens (2-OHE_1 and 2-OHE_2) dominate the profile of estrogen metabolites in these samples, which would be expected from previous studies showing that in normal humans and animals the levels of 2-catechol estrogens are much higher than those of the 4-catechol estrogens. In addition, significant amounts of methylated catechol estrogens were detected in one sample, suggesting that this protective pathway was working. All three samples contained glutathione conjugates or their break-down products, the NAcCys conjugates. The presence of the glutathione or NAcCys conjugates suggests that DNA adducts are also formed by the catechol estrogen quinones.
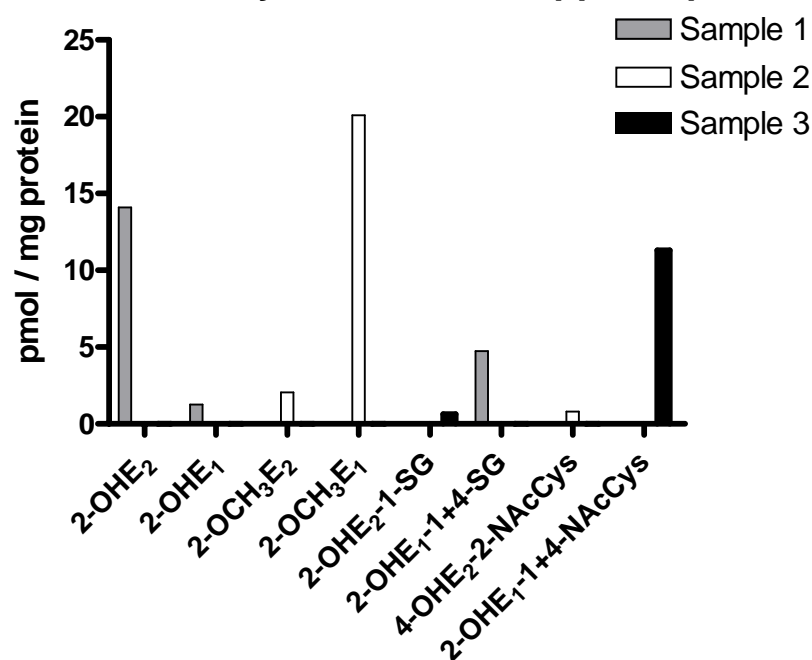

Figure 1

The proposed studies will yield valuable new information and insights in a relatively short time-frame. The analysis of estrogen metabolites, estrogen conjugates and estrogen-DNA adducts by LC/MS/MS is rapid, and sample preparation for nipple aspirate fluid is minimal (ultrafiltration to remove cells and cellular debris). The information acquired on levels of estrogen metabolites, estrogen conjugates and estrogen-DNA adducts in breast fluid will be novel, as these compounds have never before been measured in breast fluid. They should provide us with novel insights into the formation and metabolism of estrogens in the breast, the possible association with development of breast cancer and expression of selected enzymes in breast tissue. Because the subjects can be recruited and the collection of samples

completed within 12 to 18 months and the analyses can be completed soon thereafter, we expect this project to yield significant scientific progress within two years.

Similar potential estrogen biomarkers have also been observed in urine samples.

New research analyzing estrogen biomarkers in serum samples has shown that estrogen compounds can be identified in serum. These samples may provide a more extensive picture of estrogen-associated biomarkers because we know that these metabolites, conjugates and depurinating DNA adducts are excreted in both urine and feces. It is postulated that serum-based samples may be more useful in predicting cancer susceptibility.

Using the results of this study, future prospective studies can be planned where nipple aspirate samples taken from women before and after treatment with Arimidex or Tamoxifen will provide completely new information and insights into the effects of these drugs on estrogen metabolism in the breast. Other future studies include being able to determine the utility of these various estrogen derivatives as biomarkers for risk of developing breast cancer.

7. Study Population:

   a. The target population is adult women, ages 19 to 70 years of age. In 2003 the Breast Diagnostic Clinic (BDC) and Breast Cancer Clinic (BCC) there were approximately 2000 new and 3000 established patient visits. Our new patients include those seen in the BDC with new breast concerns and those at seeking counseling regarding a family history or are at high risk for developing breast cancer, as well as those women seen in the BCC with a new diagnosis of invasive or non-invasive breast cancer. Our established patients include those with a history of breast cancer, atypical hyperplasia or lobular carcinoma in situ (LCIS) and are at high risk for the development of breast cancer, and women returning for follow-up of breast concerns.

   b. The collaborative study will include women who are at elevated risk for the development of breast cancer determined by the Gail Model or a personal history of atypical hyperplasia or LCIS as well as women newly diagnosed with invasive or non-invasive breast cancer. The Gail Model, a validated tool used to estimate a woman's risk of breast cancer, incorporates five significant prognostic indicators of risk for breast cancer: age, age at menarche, number of pregnancies, number of first degree relatives with breast cancer, number of previous breast biopsies and a history of atypia on prior breast biopsy. A 5 year risk score of >1.66 % is and lifetime risk of > 20 % are considered high risk. The tool however has some limitations as it can underestimate risk for women with multiple affected relatives and those relatives diagnosed at a < age 50 years who may be BRCA 1 or 2 gene mutation carriers.   Women at high-risk are advised regarding various risk-reduction strategies such as chemoprevention with tamoxifen and even prophylactic mastectomy. Tamoxifen, a selective estrogen receptor blocker, is an approved therapy for the reduction of breast carcinoma risk. It has been shown to reduce the risk of the development of breast cancer by 49% in the Breast Cancer Prevention Trial (BCPT-P1). However, many women who are candidates for this drug choose not to take it because of side-effects which include exacerbation of menopausal symptoms and risk of thromboembolic events and endometrial carcinoma. It is becoming more important that we need to be able to provide women with an accurate risk estimate which can assist in decision-making regarding which option to pursue for risk reduction. The study of nipple aspirate fluid, urine and serum for analysis of estrogen metabolites hopefully will be able to more accurately identify women at high risk where specific modalities for risk-reduction can potentially offer the best outcome.

Potential participants will be identified by the physicians/ providers who work in the two breast clinics. Once identified the participant will meet with the study coordinator who will discuss the study, review the consent form and obtain consent from those eligible and wanting to participate in the study.

Add 1   We estimate an accrual of 160 total participants of which 80 women who are at elevated risk for breast cancer and 80 women who are newly diagnosed with breast cancer in a two-year period. The nipple aspirate fluid collected from the consenting participants will
Add 3   then be sent to Dr. Rogan at the Eppley Institute for Research in Cancer in Nebraska. Of the 160 women who consent to the study we know that that there will be women from whom we are unable to obtain nipple aspirate fluid and they will be designated as a non-yielder. Approximately 2/3 of women will produce nipple aspirate fluid, we expect to
Add 1   accrue 110 women who are able to yield nipple aspirate and 50 women who are non-yielders.

Add 1   Each participating woman will be asked to give a urine sample. If the woman chooses to have the nipple aspirate procedure performed, she will ideally be asked to provide a morning spot urine sample on the same or next day as the procedure. The urine samples will also be analyzed by ultraperformance liquid chromatography monitored by tandem mass spectrometry for the estrogen metabolites, conjugates and depurinating DNA adducts. The urine will need to be prepared with 100 mg of ascorbic acid per 50 ml sample. The ascorbic acid needs to be added before freezing. The urine samples will
Add 3   then be sent to Dr. Rogan.

Add 3   Eligible women will have the option to provide both the urine sample and have the nipple aspirate fluid procedure performed or just provide the urine sample. We will ask participants if they would like to consent to providing a single serum sample as part of this current protocol. The serum would be collected on the same day as the urine collection and/or the nipple aspirate fluid procedure.

Add 3   The number we expect to accrue who meet the eligibility criteria and provide urine and serum samples only will be 200 women over 2 years. We will plan to accrue 100 women who are at high risk and 100 women with a personal history of breast cancer.

Participant charts will be reviewed to obtain information about age, race, general health, any endocrine disorders, history of cancer, estrogen and progesterone receptor status, menopausal status, history of breast disease and medication use. If possible, the participant's history of smoking and alcohol consumption will be obtained. Such information would be relevant because alcohol and some components of smoke can induce higher levels of certain enzymes, in particular cytochrome P450s that are involved in estrogen metabolism. Subjects may be asked to provide clarification of information or information needed that is not available from their medical records.

c. There are no enrollment restrictions based on race or ethnic origin.

Inclusion Criteria
- Women between the age of 19 and 70 years of age
- Gail model 5 year score of >1.66 % (the Gail Model has been developed only for use in women 35 years or older) OR a Gail Model lifetime risk estimate of >20 % (in women younger than 35 years the 5 year risk is often very low despite significant risk factors)

- History of lobular carcinoma in-situ or atypical ductal or lobular hyperplasia (Gail model score is not necessary in women who carry these diagnoses). The nipple aspirate fluid would be obtained from the <u>unaffected breast</u>.
- Women newly diagnosed (within 30 calendar days of diagnosis) with Stages I and II (node-negative and node-positive) breast cancer <u>from the unaffected breast</u>
- Women with newly diagnosed DCIS (Stage 0) <u>from the unaffected breast</u>

<u>Exclusion Criteria</u>
- Women treated with chemotherapeutic agents for breast or other cancers
- Women with advanced breast cancer
- SERM use (Tamoxifen or Raloxifene) or Aromatase Inhibitor use
- Estrogen or other hormone use currently or in the past 3 months
- Oral contraceptive use in the past 3 months

Add 3

8. Protocol Design

This will be a prospective study.

a. **Subject Identification:** To maintain confidentiality, subjects will be identified by a code number based on the sample number and sample acquisition date and no patient identifiers will be sent to the Eppley Institute. All patient identifier information will remain at Mayo Clinic.

b. **Review of Records:** Yes, patient medical charts will be reviewed.

c. **Recruitment Process:**

Participants with an elevated risk for breast cancer and those who are newly diagnosed with breast cancer will be recruited from the BDC and BCC at the Mayo Clinic in Rochester, MN.

The control group or healthy women will be accrued at University of Nebraska Medical Center.

d. **Informed Consent Process:** When potential patients are identified they will be informed about this study by the study coordinator who will explain the study, consent form. Eligible participants are invited to participate by allowing a nipple aspirate fluid sample to be collected for use in the study. The participant will be given the option to provide a urine and serum sample in addition to the nipple aspirate fluid sample, or she can provide only urine and serum samples. The potential participant will be given the written informed consent document and will be given enough time to read and understand it. The study coordinator or investigators will be able to obtain consent.

Add 3

When the process of informed consent has been completed, the investigator will again be available to answer any questions before the participant signs the consent form. After the participant has signed the consent form the nipple aspiration procedure will be scheduled if agreed to and the urine and serum samples will be obtained.

Add 3

e. **Subject Assignment:**

Eligible participants include: 1) women at increased risk for the development of breast cancer and have a Gail model 5 year score of >1.66 % OR lifetime risk estimate of >20 % 2) personal history of atypical hyperplasia or LCIS 3) newly diagnosed with breast cancer

(within 30 calendar days) with Stages I and II breast cancer (node-negative and node-positive) 3) newly diagnosed with DCIS (Stage O)

f. **Subject Screening Procedures:**

Women who are evaluated in the BDC and BCC who are eligible participants include: 1) women at increased risk for the development of breast cancer and have a Gail model 5 year score of >1.66 % or lifetime risk estimate of >20 % 2) personal history of atypical hyperplasia or LCIS (a Gail Model score is not necessary if an individual is diagnosed with a history of atypia) 3) newly diagnosed with breast cancer (within 30 calendar days) with Stages I and II breast cancer (node-negative and node-positive) 4) newly diagnosed DCIS (Stage 0).

Women with advanced breast cancer or other disease that may profoundly alter their estrogen metabolism will not be included. Women treated with chemotherapeutic agents for breast or other cancers will also be excluded.

g. **Data Collection Procedures:**

A research study coordinator will be involved in the reviewing the participant's chart and data collection. A participant's medical chart will be reviewed to obtain information about age, race, age at menarche, number of pregnancies, number of first degree relatives with breast cancer, number of previous breast biopsies and history of atypia on prior breast biopsy, general health, endocrine disorders, menstrual status, history of cancer or prior breast disease, estrogen and progesterone receptor status of tissue in patients with prior breast cancer, tobacco, and alcohol use. Most of this information can be obtained most easily from the medical record chart.

Add 1

Once the participant has consented to the study they will be scheduled for a nipple aspirate procedure to be performed by a health care provider at the Breast Clinic, who will process the fluid for transport to the Eppley Institute in Nebraska.

Collection of nipple aspirate fluid samples will be carried out in the using the First Cyte Breast Aspirator (Cytyc Health Corporation). Although the utility of the analytical data obtained from nipple aspirate samples is not yet established, we think that we are establishing a core set of data from human [11] and animal [12-14] studies that will enable us and others to interpret the profiles of estrogen compounds in the nipple aspirate fluid samples in terms of risk of developing breast cancer.

Add 3

For subjects who have menstrual cycles, nipple aspiration will be conducted mid-cycle to obtain as uniform a set of samples as possible. The area of the skin around the nipple will be washed to reduce the possibility of infection. EMLA, an anesthetic cream, will be applied to the breast/areolar region that is to be sampled. The breast will then be warmed for 5-10 min with a heating pad and then gently massaged by the participant for 2 min. The area of the skin around the nipple will be washed to reduce the possibility of infection. An aspirator will then be centered directly over the nipple and areolar region to elicit the nipple aspirate fluid. Aspiration will be achieved by applying gentle suction using a 35 cc syringe connected to the aspirator, a technique similar to using a breast pump for lactation. The gentle suction using a 35 cc syringe draws tiny amounts of fluid from the ducts to the surface of the nipple for collection in a glass capillary tube. The amount of nipple fluid collected will be approximately 10-30 ul.

Following collection, the fluid will be chilled in ice until stored at the –80 EC prior to sending to Dr. Rogan in Nebraska. Dr. Rogan's team will analyze the nipple aspirate

fluid per protocol. This fluid will first be passed through a filter to remove cells and cell debris. The material collected on the filter will be used to prepare RNA for analysis of enzyme expression by the Molecular Biology Core. The nipple aspirate fluid will be analyzed for 31 estrogen metabolites, conjugates and depurinating DNA adducts by HPLC with electrochemical and mass spectrometric detectors.

From the experience of others, we expect 10-30 ul of nipple fluid will be obtained from each subject [7-10]. The minimum amount of nipple fluid needed for analysis is 5 ul. The analysis will be normalized on total protein concentration. Our experience to date with biological samples makes us confident that we will successfully analyze almost all of the nipple aspirate fluid samples for the estrogen compounds by using our HPLC with the mass spectrometry detector. Additional nipple aspirate fluid obtained will be stored at Mayo Clinic for future analysis that may be of value.

Previous studies of breast tissue [11] and breast fluid [15] suggest that the analysis of estrogen compounds in the breast fluid will be very informative. Based on our previous results [11], we expect to find that women with breast cancer have relatively higher levels of estrogens, more 4-CE than 2-CE, relatively lower levels of methoxy CE and higher levels of CE-GSH conjugates compared to women without breast cancer. We expect to use these data (and other data we are collecting) to develop profiles of biomarkers for women at high risk of breast cancer or protected from breast cancer. Although the absolute levels of these compounds will greatly vary, we expect that relative values of the various biomarkers will be very informative.

Add 1

The urine samples will also be analyzed by ultraperformance liquid chromatography monitored by tandem mass spectrometry for the estrogen metabolites, conjugates and depurinating DNA adducts. The urine will need to be prepared with 100 mg of ascorbic acid per 50 ml sample. The ascorbic acid needs to be added before freezing. The urine
Add 3
samples will then be sent to Dr. Rogan.

Add 3

The serum samples will need to be prepared with 1 mg of ascorbic acid per 1 ml of serum. The ascorbic acid needs to be added before freezing the sample at -80°C. 20 ml of blood will be collected for each sample in order to obtain an end product of 8 ml of serum. The samples will be processed at room temperature for optimum result. The serum will be divided into four-2 ml aliquots. Two aliquots will be shipped to Dr. Rogan and two aliquots will be stored at Mayo Clinic Rochester. Samples will be batched and shipped on dry ice.

All samples (including nipple aspirate fluid, urine and serum) are to be shipped by courier to the following address:

> Eleanor Rogan, Ph.D.
> Eppley Research Institute, ESH 6031
> University of Nebraska Medical Center
> 668 South 41st Street
> Omaha, NE 68105
> (402) 559-4095

h. **Clinical Assessments:** None will be conducted for this study.

i. **Research Interventions:** First, the participant will be asked to participate in the study
Add 1
and sign an informed consent document. Second, the participant will undergo the nipple aspiration procedure at a scheduled time. This procedure is described in detail in Section

8.f. above. The participant may also opt to have both urine and serum samples taken in addition to the nipple aspiration procedure at this time, or choose to complete the urine and serum samples alone. A day after the nipple aspiration procedure, the study coordinator or health care provider will call the participant and ask how she is feeling and whether any problems have arisen. Assuming that none have arisen, that will be the last event the participant will experience as part of this study.

j. Data Analysis:

For all participants, characteristics including race, body mass index, age at menarche, menopausal status, age at menopause if applicable, smoking history, alcohol consumption, pregnancy history including age at each pregnancy, lactation, history of benign breast disease, hysterectomy and disease type (for breast cancer patients) will be collected. Our initial analyses of urine samples have not shown differences in the levels of estrogen-DNA adducts between pre- and post-menopausal women. We will have both pre- and post-menopausal women in this study and will learn whether this preliminary finding holds true in the larger data set. All hypothesis tests will be two-sided and conducted at the 0.05 level of significance. Participant characteristics will be summarized using descriptive statistics.

Statistical considerations for Group 1
The comparisons of interest are the levels of estrogens, catechol estrogen metabolites, catechol estrogen-GSH conjugates and catechol estrogen-DNA adducts (N3Ade and N7Gua) among healthy women, high risk women and women with breast cancer. The three groups will be matched on age (19-34, 35-50, 51-70). Analysis of variance (ANOVA) will be used to test whether there is a significant difference in the mean analytes among the groups. If ANOVA indicates a significant difference in between the means, further analysis will be conducted using Tukey's pairwise comparison procedure to control for multiple testing. The distribution of the patient characteristics described above among the three groups will be examined. Multivariate regression will be used to adjust for relevant patient characteristics that do not appear to be balanced among the groups.

Our first study of breast samples (~1 g each) obtained from breast biopsies, which included 49 control women (18 with normal tissue and 31 with fibrocystic changes) and 28 with breast carcinoma, yielded statistically significant differences between cases and controls in the levels of 4-OHE$_1$(E$_2$) ($p<$ 0.01), and E$_1$(E$_2$)-Q conjugates ($p<$ 0.003) [8], although the analyses were much less sensitive at that time. These results strongly suggest that the analyses of nipple aspirate fluid samples will provide even more significant data. We therefore anticipate a medium effect size of 0.20 [40]. These data suggest a difference of approximately 0.64 standard deviations between breast cancer cases and healthy controls. Assuming that the means for healthy controls, high risk and breast cancer participants are approximately equally spaced in this range, a 0.05 level of significance and 80% power, 80 subjects with nipple aspirate fluid available for analysis in each group are needed [40]. A total of 100 subjects in each of the three groups will provide approximately 80% power (at the 0.05 level of significance) to detect an effect size of 0.18 in the mean analytes obtained from urine and serum samples.

9. Risks/Benefits Assessment

   a. Risks: The potential risks from this procedure include possible pain associated with the procedure. Participants will be advised that the results from this study will be combined with the results of other studies to learn what factors are significant in the development of breast cancer, and these results cannot be used by themselves to predict risk of developing breast cancer.

Add 1   To address possible physical and/or emotional risks, at the time of the procedure the study coordinator will provide subjects with telephone numbers if they have problems or concerns, and she will contact the subjects by telephone a day or so after the procedure to inquire if there are an symptoms after the procedure. If a subject is having problems or concerns, Dr. Pruthi will be informed and will arrange for the participant to receive appropriate medical care as soon as possible.

   b. Benefits to the subject: There will be no direct benefit to the subjects from the analyses of estrogen compounds.

Add 3   **c. Compensation:** Participants will receive $25.00 remuneration for their participation in the nipple aspirate fluid collection component of the study.

10. Reporting of Serious of Unexpected Adverse Events: A serious adverse event would be an undesirable experience associated with undergoing nipple aspiration that resulted in death, a life-threatening condition, hospitalization, disability or intervention to prevent permanent impairment or damage. Nipple aspiration has been conducted for 20 years [7-10]. Thus, the occurrence of serious adverse events is highly unlikely and the procedure is very similar to using a breast pump for lactation.

11. Description of Protocol Drugs or Devices: Not applicable.

   a. Disposition of Data: Data will be stored in the Eppley Institute (offices 6006 and 6015, Eppley Science Hall) in locked cabinets and in computer files protected by passwords. The information about a subject will be stripped of its identifiers by the staff member maintaining our database and assigned a code number based on sample number and date of acquisition, which will allow us to refer back to the medical record, through the nurse coordinator, if needed. The code breaker document is kept in Dr. Rogan's laboratory in paper form in a locked file cabinet in room 6015 ESH, the office of Ms Sheila Higginbotham, Research Technologist II, who maintains all our human subjects' records. Ms Higginbotham has the key to the cabinet. The code breaker document is kept electronically in Ms Higginbotham's password-protected human subjects' folder on the Eppley Institute Local Area Network. Data coded to protect subjects= identities will be used internally by members of the research group only for statistical analysis of the results of the determinations of estrogen compounds in the nipple aspirate fluid samples. One of the strengths of our proposal is the ability to use subject information (obtained from patient records at Mayo Clinic, Rochester, MN) on age, race, smoking history, alcohol consumption, menopausal status, hormone receptors, etc., in analyzing the results of the study. Reports and publications resulting from this study will not contain any information that identifies subjects.

12. Modification of the Protocol: Any modifications to the protocol and/or consent form will be submitted to our IRB and Cancer Center Clinical Research Administrative Committee for approval.

13. Departure from the Protocol: Any deviations from the protocol, which are not expected, will be reported to our IRB and Cancer Center Clinical Research Administrative Committee for approval.

14. Medical Care for Research-Related Injuries:

To address any concerns at the time of the procedure, the study coordinator will provide subjects with telephone numbers to call. The RN in the GCRC will contact the subjects by telephone a day or so after the procedure to inquire about whether they are having any medical concerns after the procedure.

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MOLECULAR BIOLOGY CORE - SUTTER

Approved Work statement for Year 3

Perform microarray analyses and RealTime PCR measurements on samples from animals and cell culture.

Progress

Introduction

The molecular biology core of this BCCOE brings high throughput molecular analyses to the specific aims of this center. The primary capacity of this core is high throughput gene expression analysis facilitated by Affymetrix GeneChip technology. In addition to massively parallel analysis of gene expression, new technologies, released in 2005, permitted the expansion of these facilities into high resolution analysis of chromosome structure. As decided at the December 6, 2004 meeting of the BCCOE held in Washington, DC, the efforts of this core in Year 2 focused on **Specific Aim 2 (Russo)** of the center: **to determine the effects of estrogen and its metabolites on the progressive steps of neoplastic transformation of human breast epithelial cells (HBECs) and to determine whether the neoplastic phenotypes and genotypes thus induced can be abrogated by known and new preventive agents.**
Methods and procedures

The Russo and Sutter laboratories have been actively collaborating on the molecular characterization of events that occur during the progressive malignant transformation of MCF-10F cells.

Cell Lines, DNA and RNA

The cell lines and the malignant transformation protocol are shown in Fig. 1 and described previously. In this work, three individual samples of each cell line were analyzed as independent replicates. For comparison to the previous work we report the current and (previous) cell sample designations: MCF-10F samples 1,2 3 (MCF-10F 1,2,3); trMCF samples 1,2 3 (E_2-70 nM 1,2,3); bsMCF (C5 1,2,3) and caMCF (L1, L4, L8). The designation E_2-70 nM referred to treatment conditions resulting in cell transformation; C5 referred to the position of the well in the selection chamber; L1, L4 and L8 referred to the cell line derived from the tumor of C5 cells in animal 1 (L1) and so forth.   For isolation of the bcMCF cell lines, bsMCF cells were plated at low density and observed under the microscope. Individual colonies were isolated using cloning rings, selective trypsinization and plating, giving rise to six clones designated Clones A, B, C, F, H, and I. bcMCF clones A, B and F were analyzed in this study. Furthermore, the Clone A cell line of bcMCF was tested for its tumorigenic capacity in 45-day-old female SCID mice, which were obtained from the Fox Chase Cancer Center animal care facility, as previously described. Animals were housed four to a cage and maintained in a laminar flow rack at 72 °F with a 12h light/dark cycle. They received water and food *ad libitum*. The cells were injected into the mammary fat pad of the abdominal region of the mice at a concentration of 10-15 x 10^6 cells suspended in 0.1 ml of sterile phosphate buffered saline. The animals were palpated twice a week for detection of tumor development and were followed for up to six months post injection. Animals were killed by carbon dioxide inhalation. Each animal was autopsied, carefully examined for identification of visceral metastasis and palpable tumors were dissected from the skin. High molecular weight genomic DNA and total cellular RNA were isolated from three individual samples of each cell line: MCF-10F, trMCF, bsMCF,

bcMCF, and caMCF. For DNA, cells were treated with lysis buffer containing 20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 25 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulphate, and 200 μg/mL proteinase K for 15 min at 65 $^{\circ}$C with gentle agitation. The samples were cooled on ice and treated with 100 μg/mL RNase A at 37 $^{\circ}$C for 30 min. The samples were extracted once with buffered phenol, and again with chloroform:isoamyl alcohol (24:1). The aqueous layer was adjusted to 0.75 M ammonium acetate and the DNA was precipitated by the addition of 2.5 volumes of 100% ethanol. The precipitate was washed with 70% ethanol, dried and dissolved in sterile water. Total cellular RNA was isolated using the TRIZOL (Life Technologies, Gaithersburg, MD) modification of the guanidinium thiocyanate procedure. The concentration and quality of the DNA and RNA was determined spectrophotometrically and by capillary gel electrophoresis (Agilent 2100 Bioanalyzer, Palo Alto, CA).

Genotyping and microarray assays

Affymetrix 100k Single Nucleotide Polymorphism (SNP) mapping was performed using the combined *Xba* I (Mapping50K_Xba240) *Hin*d III (Mapping50K_Hind240) GeneChip mapping Array set according to the manufacturer's recommended procedures (Affymetrix, Santa Clara, CA) with the following modifications. The time of the restriction endonuclease digestions was increased to 6 hr at 37 °C, and the ligation reaction was carried out overnight at 16 °C. Using this 100k SNP set, one obtains allele information at a mean intermarker distance of 23.6 kb and median intermarker distance of 8.5 kb. The average heterozygosity of each SNP is 0.30. Gene expression microarray analysis was performed using the Affymetrix HG-U133_Plus_2 Array, measuring more than 47,000 transcripts. Eight μg of total RNA was used in the cDNA synthesis reaction. After hybridization, the chips were washed and scanned on the GeneChip Scanner 3000 (Affymetrix). The genotype calls (heterozygous or homozygous) were determined using the Affymetrix GTYPE v4.0; the P(Present)- or A(Absent)-calls of the probes in the gene expression chips were determined using the Affymetrix GCOS v1.4. The microarray data have been submitted to the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus repository (series accession no. GSE**XXX**; or sample IDs **GSMXX–GSMXX**).

Data analysis

The chromosome copy number changes and LOH were determined using dChip (20). The .CEL files of Mapping50K_Hind240 and Mapping50K_Xba240 chips and their corresponding .TXT files containing the SNP genotype calls were put into dChip to calculate the intensities of probes. The human genome release v17 was used to provide the genome information files (refGene and cytoBand files) that were used for the SNP data analysis in dChip. The output files containing the SNP intensities and SNP genotype calls were merged together for the 100k SNP analysis. The MCF-10F cell line served as the diploid reference for detection of copy number changes. The genotype of the MCF-10F cell line was also interrogated using the Affymetrix CNAT v3.0, in order to detect potential aneuploidy. Genomic smoothed analysis (GSA) with 0.5Mbp distance was used to delineate the copy number change and LOH. The intensities of probe sets in the HG-U133_Plus_2 Genechips were calculated by dChip software using the Perfect-match/Mismatch difference model after invariant-set normalization. A gene is considered expressed in the group of interest if the gene is "Present" in all 3 samples of that group. Differentially expressed genes were identified by pairwise comparison using MCF-10F as the reference. The significance level was $p < 0.05$ in an unpaired t-test of the log transformed expression values. To identify and extract the expression data for probesets corresponding to genes located within specific regions of individual chromosomes, the Entrez Gene ID and corresponding location in Mb were used to search the annotation file of the HG-U133_Plus_2

Genechip. To integrate the Entrez Gene ID and associated gene with the human genome map, we used the GeneLoc tool of the Weismann Institute of Science.

Proposed plan of research
        In year 4, we will publish two papers on our collaborative analysis describing progression of breast cell transformation in (Specific Aim 2). In the first publication, we will describe the alterations in chromosome structure identified above. In a second manuscript, we will combine chromosome structure analysis with RNA microarray analysis in order to understand the cell biology of malignant transformation. We will expand these studies into the areas of biomarker development, breast cancer prevention, and the cancer biology of estrogen receptor-negative breast cancer.

Key research accomplishments
        Using a well characterized *in vitro-in* vivo model of breast carcinogenesis, we have demonstrated the ability to characterize cell transformation at the combined levels of the complete genome and the individual gene. This research should lead to vastly improved understandings of cell transformation and tumorigenesis and permit translational studies to human breast cancer cases.

Reportable outcomes
1.   Rahman, M.M., Sutter, C.H., Emmert, G.L., and **Sutter, T.R.** Regioselective 2-hydroxylation of 17β-estradiol by rat cytochrome P4501B1. *Toxicol. Appl. Pharmacol.,* in press, 2006.

2.   **Sutter, T.R.**, Fernandez, S.V., Huang, Y., Goodwin, Y., Russo, P.A., Russo, I.H., and Russo, J. Gemomic profiles of estrogen induced malignant transformation of MCF-10F cells reveal early events in breast cancer progression. Sumitted, 2006

Abstract:
Estrogen is a risk factor for breast cancer, yet its mechanism in the initiation of this disease is not clear. Here we use a model of estrogen-mediated malignant transformation of MCF-10F cells to identify the temporal acquisition of changes in genome structure and gene expression that correspond to the progressive transformed phenotype culminating in tumorigenesis. Genomic DNA and total RNA were isolated from MCF10F, trMCF (MCF10F transformed by 70 nM 17-beta estradiol), bsMCF (trMCF selected by Boyden Chamber, tumorigenic), bcMCF(clones of bsMCF), and caMCF (cells from tumors of bsMCF grown in SCID mice). The Affymetrix 100k SNP and HG-U133_Plus_2 chips were used for genotyping and gene expression analyses. Changes in chromosomal copy number and loss of heterozygosity were progressive. Gross changes were rarely observed in the trMCF10F; the earliest was a gain in chromosome 1p, 1p36.12-1p36.21. In the bsMCF and their sub-clones bcMCF, additional gains were seen in chromosomes 1p, 1p36.12-1pter, 5q, 5q21.1-5q35.3, and 13q, 13q21.32-13q34; losses were detected in chromosomes 4 and 8p, 8p11.1-8p23.1. In the caMCF, additional losses were seen in chromosomes 3p, 3p12.1-3p14.1, 9p, 9p22.1-9p24.3, and 18q, 18q11.2-18q23. Using microarray analysis we were able, in some cases, to narrow the regions of interest and their associated candidate cancer genes. The observation of progressive genomic changes, along with the tumor phenotype of a poorly differentiated adenocarcinoma, indicate an underlying mechanism common to cancer development in humans, and adds to the evidence indicating that estrogens are mutagenic and contribute early to the process of breast cancer.

Conclusions

We have established a strong and functional collaboration with other investigators in the BCCOE. Our studies show important new aspects of the progression of cell transformation leading to tumorigenesis *in vivo*, and demonstrate our ability to move effectively between studies of chromosome structure and gene function.

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Genomic Profiles of Estrogen Induced Malignant Transformation of MCF10F Cells Reveal Early Events in Breast Cancer Progression

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Abstract

Estrogen is a risk factor for breast cancer, yet its mechanism in the initiation of this disease is not clear. Here we use a model of estrogen-mediated malignant transformation of MCF10F cells to identify the temporal acquisition of changes in genome structure and gene expression that correspond to the progressive transformed phenotype culminating in tumorigenesis. Genomic DNA and total RNA were isolated from MCF10F, trMCF (MCF10F transformed by 70 nM 17-beta estradiol), bsMCF (trMCF selected by Boyden chamber, tumorigenic), bcMCF(clones of bsMCF), and caMCF (cells from tumors of bsMCF grown in SCID mice). The Affymetrix 100k SNP and HG-U133_Plus_2 chips were used for genotyping and gene expression analyses. Changes in chromosomal copy number and loss of heterozygosity were progressive. Gross changes were rarely observed in the trMCF10F cells; the earliest was a gain in chromosome 1p, 1p36.12-1p36.21. In the bsMCF and their sub-clones bcMCF, additional gains were seen in chromosomes 1p, 1p36.12-1pter, 5q, 5q21.1-5q35.3, and 13q, 13q21.32-13q34; losses were detected in chromosomes 4 and 8p, 8p11.1-8p23.1. In the caMCF, additional losses were seen in chromosomes 3p, 3p12.1-3p14.1, 9p, 9p22.1-9p24.3, and 18q, 18q11.2-18q23. Using microarray analysis we were able, in some cases, to narrow the regions of interest and their associated candidate cancer genes. The observation of progressive genomic changes, along with the tumor phenotype of a poorly differentiated adenocarcinoma, indicate an underlying mechanism common to breast cancer development in humans, and adds to the evidence indicating that estrogens are mutagenic and contribute early to the process of breast cancer.

Introduction

Breast cancer accounts for up to one-third of all new cases of women's cancer in North America and is the most frequently occurring cancer in females worldwide. In the United States alone, more than 200,000 invasive cases will be diagnosed this year. While its incidence continues to rise, the mortality rate for breast cancer has remained unchanged in the past five decades, placing it as the leading cause of cancer-related death in non-smoking women (1, 2).

While germ line mutations in genes including BRCA1 and BRCA2 confer a strong predisposition in familial cases of breast cancer, such genes account for only 5-10 percent of the occurrence of all cancers at this site. Therefore, the majority of risk for developing breast cancer can be attributed to unknown polygenic predisposition and the interaction of these gene effects with environmental factors (3). One such gene-interacting factor is estrogen. Compelling evidence indicates that lifetime exposure to estrogen is an important determinant of the risk of developing breast cancer. The weight of evidence implicating estrogen in the etiology of breast cancer includes both risk factors that affect cumulative estrogen exposure, including early menarche, late menopause, alcohol consumption, post-menopausal obesity and hormone replacement therapy, as well as direct associations of risk with increased blood estradiol concentrations (4, 5). These observations in humans are strongly supported by experimental studies in animals that consistently show that estrogens are potent mammary carcinogens and that these effects can be ablated by ovariectomy or the administration of anti-estrogenic chemicals (4).

However, despite this knowledge, the molecular mechanisms underlying the development of estrogen-associated breast cancer are not completely understood. There are three mechanisms considered responsible for the carcinogenicity of estrogens: i) receptor-mediated hormonal

activity, which stimulates cellular proliferation, resulting in more opportunities for accumulation of the genetic damage that leads to cancer; ii) cytochrome P450-mediated metabolic activation, which elicits direct genotoxic effects and increases mutation rates; and iii) the induction of aneuploidy by estrogen (4-8).

The carcinogenic action of estrogen on human breast epithelial cells (HBECs) was demonstrated, *in vitro*, by estrogen-mediated transformation of the spontaneously immortalized HBEC line, MCF10F. Treatment of these cells with either 17β-estradiol (E$_2$) or its DNA reactive catechol metabolites resulted in acquisition of transformed phenotypes including colony formation in agar methocel, decreased ductulogenesis, and increased invasiveness (9). Of great interest, MCF10F cells do not have detectable levels of expressed estrogen receptor alpha (ERα) and the *in vitro* cell transformation was not abrogated by the co-treatment of these cells with the antiestrogen ICI-182-780, supporting a non ERα-mediated mechanism (10).   Recently, complete neoplastic transformation of MCF10F cells was demonstrated by the formation of tumors in an appropriate heterologous host (11). MCF10F cells transformed with 70 nM E2 and selected for an invasive phenotype by growth through a matrigel coated Boyden chamber formed tumors when injected into the mammary fat pad of the abdominal region of severe combined immune depressed (SCID) mice. Cell lines established from these tumors also formed tumors in SCID mice (11); Figure 1). The tumors were poorly differentiated adenocarcinomas characteristic of primary breast tumors (11).

As a model of estrogen-mediated malignant transformation of human breast, this is a unique system for identifying the temporal acquisition of changes in genome structure and gene expression that correspond to the progressive transformed phenotype culminating in tumorigenesis. Here we report on concurrent genomic changes in copy number and loss of

heterozygosity (LOH) that occur during MCF10F cell transformation showing the occurrence of specific and progressive genomic changes indicative of mechanisms common to cancer development in humans.

Materials and Methods

Cell Lines, DNA and RNA

The cell lines and the malignant transformation protocol are shown in Fig. 1 and described previously (11). In this work, three individual samples of each cell line were analyzed as independent replicates. For comparison to the previous work we report the current and (previous) cell sample designations: MCF10F samples 1,2 3 (MCF10F 1,2,3); trMCF samples 1,2 3 (E_2-70 nM 1,2,3)]; bsMCF (C5 1,2,3) and caMCF (L1, L4, L8). The designation E_2-70 nM referred to treatment conditions resulting in cell transformation; C5 referred to the position of the well in the selection chamber; L1, L4 and L8 referred to the cell line derived from the tumor of C5 cells in animal 1 (L1) and so forth (11). For isolation of the bcMCF cell lines, bsMCF cells were plated at low density and observed under the microscope. Individual colonies were isolated using cloning rings, selective trypsinization and plating, giving rise to six clones designated Clones A, B, C, F, H, and I. bcMCF clones A, B and F were analyzed in this study (bcMCF1, bcMCF2 and bcMCF3 respectively). Furthermore, the Clone A cell line of bcMCF was tested for its tumorigenic capacity in 45 day old female SCID mice, as previously described (11). High molecular weight genomic DNA and total cellular RNA were isolated from three individual samples of each cell line: MCF10F, trMCF, bsMCF, bcMCF, and caMCF. For DNA extraction we use the same protocol described elsewhere (11). Total cellular RNA was isolated using the TRIZOL (Life Technologies, Gaithersburg, MD). The concentration and quality of the DNA and

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RNA was determined spectrophotometrically and by capillary gel electrophoresis (Agilent 2100 Bioanalyzer, Palo Alto, CA).

Genotyping and microarray assays

Affymetrix 100k Single Nucleotide Polymorphism (SNP) mapping was performed using the combined *Xba* I (Mapping50K_Xba240) *Hin*d III (Mapping50K_Hind240) GeneChip mapping Array set according to the manufacturer's recommended procedures (Affymetrix, Santa Clara, CA) with the following modifications. The time of the restriction endonuclease digestions was increased to 6 hr at 37 $^\mathrm{o}$C, and the ligation reaction was carried out overnight at 16 $^\mathrm{o}$C. Using this 100k SNP set, one obtains allele information at a mean intermarker distance of 23.6 kb and median intermarker distance of 8.5 kb. The average heterozygosity of each SNP is 0.30. Gene expression microarray analysis was performed using the Affymetrix HG-U133_Plus_2 Array, measuring more than 47,000 transcripts. Eight μg of total RNA was used in the cDNA synthesis reaction. After hybridization, the chips were washed and scanned on the GeneChip Scanner 3000 (Affymetrix). The genotype calls (heterozygous or homozygous) were determined using the Affymetrix GTYPE v4.0; the P(Present)- or A(Absent)-calls of the probes in the gene expression chips were determined using the Affymetrix GCOS v1.4. The microarray data have been submitted to the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus repository (series accession no. GSE**XXX**; or sample IDs **GSMXX–GSMXX**).

Data analysis

The chromosome copy number changes and LOH were determined using dChip (12). The .CEL files of Mapping50K_Hind240 and Mapping50K_Xba240 chips and their corresponding .TXT

files containing the SNP genotype calls were put into dChip to calculate the intensities of probes. The human genome release v17 was used to provide the genome information files (refGene and cytoBand files) that were used for the SNP data analysis in dChip. The output files containing the SNP intensities and SNP genotype calls were merged together for the 100k SNP analysis. The MCF10F cell line served as diploid reference for detection of copy number changes. The genotype of the MCF10F cell line was also interrogated using the Affymetrix CNAT v3.0, in order to detect potential aneuploidy. Genomic smoothed analysis (GSA) (13) with 0.5Mbp distance was used to delineate the copy number change and LOH. The intensities of probe sets in the HG-U133_Plus_2 Genechips were calculated by dChip software using the Perfect-match/Mismatch difference model after invariant-set normalization. A gene is considered expressed in the group of interest if the gene is "Present" in all 3 samples of that group. Differentially expressed genes were identified by pairwise comparison using MCF10F as the reference. The significance level was $p < 0.05$ in an unpaired t-test of the log transformed expression values. To identify and extract the expression data for probe sets corresponding to genes located within specific regions of individual chromosomes, the Entrez Gene ID and corresponding location in Mb were used to search the annotation file of the HG-U133_Plus_2 Genechip. To integrate the Entrez Gene ID and associated gene with the human genome map, we used the GeneLoc tool of the Weismann Institute of Science (14).

Results

      Estrogen-mediated malignant transformation of the human breast epithelial cell line MCF10F. The experimental model used in this study is shown in Fig. 1. Five distinct cell populations, corresponding to unique phenotypes of this malignant transformation protocol, were

analyzed for changes in genome structure and gene expression. These cell lines include

MCF10F, immortal, not tumorigenic; trMCF, transformed by treatment with 70 nM E_2, not

tumorigenic; bsMCF, selected for invasiveness by growth through a matrigel coated Boyden

chamber, tumorigenic; bcMCF, sub-clones of the bsMCF cell populations isolated in this study,

tumorigenic; and caMCF, human tumor cells isolated from individual tumors produced in SCID

mice, tumorigenic.

Concurrent analysis of chromosome copy number and LOH. We used the 100k SNP

GeneChip Mapping Array set to interrogate the structure of chromosomes 1-22 and X at very

high resolution, with a mean intermarker distance of 24 kb. For each sample, more than 100,000

specific SNP sites were queried at each parental allele and genotyped as homozygous AA,

homozygous BB or heterozygous AB. No difference in the quality of the data was observed for

the between group samples. We have attempted to analyze genomic DNA isolated directly from

the tumor tissue from SCID mice, and although these samples contained high molecular weight

DNA, of equal purity to the others, we were not able to determine their genotypes. It is possible

that contamination of the human DNA with mouse DNA from infiltrating cells is responsible,

but this remains unknown. However, by establishing cell lines from the tumors, we were able to

preserve most, if not all, of the structural changes occurring during tumorigenesis.

To observe relevant changes in chromosomal copy number and regions of LOH, we

identified the MCF10F data as the reference sample(s). Our rationale for this choice is based on

the goal of identifying progressive changes in genome structure that accompany specific

phenotypic stages of malignant cell transformation. As the MCF10F cells are not tumorigenic,

those changes existing in this cell line may be predisposing but not causal in tumor formation.

Previous studies of MCF10F have detected a chromosome loss at 1p, a gain at 8q24.1 and

a translocation, t(3p13:9p22), (8, 11). As shown if Fig. 2, these copy number changes in chromosomes 1 and 8 can be detected by comparing the MCF10F data with a standard reference set. However, the current PCR- and Chip hybridization-based methods are not able to detect chromosomal translocations, and such changes occurring during malignant transformation will not be detected.

The changes in chromosomal copy number and LOH occurring throughout cell transformation are shown in Fig. 3. The data are shown for three independent replicate samples of each group and in the case of the bcMCF group, these samples are from individual cell clones. Changes in chromosomal copy number and loss of heterozygosity were progressive. Gross changes were rarely observed in the trMCF10F; the earliest was a gain in chromosome 1p, 1p36.12-1p36.21. In the bsMCF and their sub-clones bcMCF, additional gains were seen in chromosomes 1p, 1p36.12-1pter, 5q, 5q21.1-5q35.3, and 13q, 13q21.32-13q34; losses were detected in chromosomes 4 and 8p, 8p11.1-8p23.1. In the caMCF, additional losses were seen in chromosomes 3p, 3p12.1-3p14.1, 9p, 9p22.1-9p24.3, and 18q, 18q11.2-18q23. In all cases, regions of progressive LOH were accompanied by allelic imbalance (Fig.3). Of interest, regions of LOH were detected in all cell lines, including the parental line MCF10F, in regions of chromosomes 1p, 5q and 13q, where copy number gains were observed (Fig. 3). In the case of chromosome 1, this region has a deletion in the MCF10F line (Fig. 2). Therefore, the subsequent increase in copy number is occurring in a single allele and becasue this occurs early in trMCF cells, is consistent with imbalance associated with a gain of function mutant and a gene dosage effect in an oncogene involved in the earliest cell transformation phenotypes. While no changes in copy number were observed for the 5q or 13q regions in MCF10F (data not shown), the LOH

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within these regions of MCF10F suggest prior allelic imbalances. Thus, amplifications appear to occur within chromosomal regions of existing allelic imbalance.

Selection for allelic imbalance during cell transformation. This cell transformation model involves treatment of immortal MCF10F cell and selection for a transformed phenotype followed by further selection for invasiveness and then tumorigenesis (Fig.1, (11). In the cases of the trMCF and bsMCF, these phenotypes were selected from a population of cells, some of which contain genomic alterations that confer a selective advantage. As major changes in copy number and LOH were observed for chromosome 4 (Fig. 3), we present this chromosome at higher resolution. Firstly, the changes in chromosome 4 detected in bsMCF cells can be observed in samples of trMCF cells (Fig. 4*A*), albeit at a level that does not reach statistical significance. This observation is consistent with the concept that the trMCF cells, while all having transformed phenotypes of growth in agar methocel and decreased ductulogenesis, are a heterogenous cell population. Within this population, it is likely that there exists some cells with alterations in additional chromosomes, such as 4, that are selected for by growth through a matrigel coated Boyden chamber. These selected cells, bsMCF, and their subclones, bcMCF, are tumorigenic, indicating that some of these changes in genome structure are associated with malignant transformation. Furthermore, the tumor cell lines, isolated from tumors in SCID mice, contain additional regions of loss at 3p, 9p and 18q. At this time we cannot distinguish whether these changes were selected for by growth in a heterologous host or whether they occurred de novo during tumorigenesis. Secondly, in some samples (Fig. 4*B,* bsMCF1) and in some clones (Fig. 4*B,* bcMCF1) of bsMCF cells, smaller more discrete regions of loss and LOH are detected in chromosome 4. The bcMCF1 induced tumors in SCID mice (data not shown). As in this example then, the isolation of subclones affords the possibility to capture and detect minimal

regions of allelic imbalance associated with the temporal and selective aspects of malignant cell transformation.

Identification of Candidate Oncogenes and TSGs. In order to identify candidate genes, we used the SNP_ID bounding each region of chromosomal copy number change or LOH to define the physical location of the region in units of Mb. Using Entrez Gene, we then extracted the ordered list of expressed genes corresponding to each region as determined by microarray analysis. Next, we integrated this data to produce a refined list of expressed genes corresponding to each chromosomal region of interest.

As the changes in genotype and malignant phenotype were observed to be progressive throughout the transformation protocol, we analyzed the cell populations sequentially. Here we report on the changes occurring during the initial cell transformation (trMCF) and after selection for growth through a Boyden chamber and ring cloning (bcMCF). The additional deletions occurring in the caMCF cells at chromosomes 3p, 9p and 18q will be published elsewhere in a manuscript describing host-cell interactions during tumorigenesis.

In the earliest population of trMCF cells, only a single region of amplification is observed in chromosome 1p36.21-1p36.12, corresponding to the Mb range of 14.469 – 20.912. This region contains 76 genes of which 27 are expressed in the trMCF cells. In the parental MCF10F cells, this region of chromosome 1 has a loss of one allele (Fig. 2). Therefore, LOH is also observed in this same region where subsequent amplification has occurred. This type of chromosomal anomaly is consistent with a mechanism of a gain of function mutation in an oncogene with a gene dosage effect of amplification. However, as LOH is observed in this region, we must also consider the presence of candidate TSGs. Nine expressed candidate genes,

consistent with these mechanisms, or know to be associated with breast or other cancers, are identified in Table 1. (Four of these genes, identified in Table 1 by shading, are discussed below).

In the bcMCF cells, which have acquired tumorigenic potential (Fig. 1), three regions of chromosomal amplification and two regions of deletion are observed. The region of chromosome 1 amplification has expanded to include 1pter-1p36.12, corresponding to the Mb range of 0.328 – 20.912. This region contains 241 genes of which 98 are expressed in the bcMCF cells. Forty-four of these genes, meeting the criteria described above, are identified in Table 2.

A second region of amplification is observed at chromosome 5q21.1-5q35.3, corresponding to the Mb range of 101.142 - 179.773. This region contains 466 genes of which 224 are expressed. Forty genes having statistically significant increases in RNA expression levels, consistent with a gene dosage effect, are identified in Table 2. In addition, a region of LOH found in all cell lines is present at 5q33.2, corresponding to the Mb range of 148.165 – 148.670. This region contains 4 genes of which 3 are expressed. One candidate oncogene, ABLIM3, is identified Table 2.

A third region of amplification is observed at chromosome 13q21.31-13q34, corresponding to the Mb range of 62.699 – 112.940. This region contains 97 genes of which 53 are expressed. Nine genes having statistically significant increases in RNA expression levels, consistent with a gene dosage effect, are identified in Table 2. In addition, a region of LOH found in all cell lines is present at 13q33.1, corresponding to the Mb range of 78.968 – 79.278. This region contains 4 genes of which 3 are expressed; none showing statistically significant changes in RNA expression levels. A second region of LOH, corresponding to the MB range of

83.188 – 84.028 contains 2 genes, none of which are expressed. In addition, 3 genes (KLF5, LIG4 and ATPIIA) showing statistically significant decreases in RNA expression levels and known to be associated with cancer are identified in Table 2.

In the bcMCF cells, two regions of deletion are observed. The first region of deletion occurs in chromosome 4. This observed change in chromosomal copy number is accompanied by LOH, indicating a loss of a single parental allele. As described above, the bcMCF clone A (bcMCF1) cells were shown to be tumorigenic, yet these cells showed a restricted set of deletions of chromosome 4 in comparison to the other clones, showing complete loss of one copy of this chromosome. This result indicates that sub-cloning can be effective for identifying minimal regions of chromosomal instability that are associated with the tumorigenic phenotype. The candidate TSGs corresponding to each region of deletion in bcMCF clone A (bcMCF1) are presented in Table 2. Region 1 of chromosome 4 is observed at 4p16.1, corresponding to the MB range of 8.657 – 10.435. This region contains 8 genes, 2 of which are expressed and identified as candidate TSGs in Table 2. Region 2 is observed at 4q13.1-4q13.2, corresponding to the Mb range of 65.333 – 67.666. This region contains 1 gene that is not expressed. Region 3 is observed at 4q21.21, corresponding to the MB range of 79.795 – 82.427. This region contains 9 genes, 4 of which are expressed (Table 2). Region 4 is observed at 4q25, corresponding to the MB range of 108.324 – 109.815. This region contains 5 genes, 4 of which are expressed (Table 2). Region 5 is observed at 4q28.3, corresponding to the MB range of 134.451 – 135.178. This region contains no genes. Region 6 is observed at 4q31.23 – 4q32.1, corresponding to the MB range of 150.275 – 156.349. This region contains 25 genes, 10 of which are expressed (Table 2). Region 7 is observed at 4q33, corresponding to the MB range of 171.612 – 172.551. This region contains no genes.

The second region of deletion is observed for chromosome 8p23.1 – 8p11.1, corresponding to the Mb range of 12.030 – 43.322. This region contains 175 genes, 92 of which are expressed and identified as candidate TSGs in Table 2. This loss in chromosomal copy number is accompanied by LOH, indicating a loss of a single parental allele. (For each of these regions, the genes identified in Table 2 by shading are discussed below).

Discussion

By high resolution and integrated genomic analysis, we have shown that the mechanism underlying neoplastic cell transformation by E2 of the estrogen receptor alpha negative MCF-10F cells is the same as the mechanism driving cancer in the human breast, *i.e.* progressive genomic instability corresponding to a progressive tumorigenic phenotype.

The earliest events observed in the trMCF cells are consistent with the idea of a cell population characterized by a mixture of cells containing a small number of common chromosomal abnormalities (the single amplification at chromosome 1p) and a few rare events occurring within this population of phenotypically altered cells (deletions of chromosome 4). From this population of phenotypically transformed cells, a subset of cells was selected, by growth through a matrigel-coated Boyden chamber, for conferring an enhanced invasive potential. These cells produce tumors in SCID mice.

Several advantages of this cell transformation model are evident. First, progressive stages of tumorigenic capacity were observed phenotypically and genotypically, with relatively few samples. Second, it is possible to test the gene-phenotype relationship using cell transfection and knock-down technologies. Third, by ring cloning of specific cells it is possible to refine the map position of candidate oncogenes and TSGs and to determine the frequency of rare events

occurring within a cell population towards understanding stochastic influences on cell transformation.

There are also challenges to this line of research. It seems quite likely that the changes in genomic profiles observed in this study would be indicative of a subset of breast cancer, not cancer in general. Finally, as MCF10F cells represent the genetic background of a single person, this study cannot elaborate on the role of genetic variation in breast cancer susceptibility.

Reviews of the literature describe more than 15 chromosome abnormalities, some with several specific regions, occurring in breast cancer (15, 16). Very few studies have addressed the issue of subsets of breast cancer. Nonetheless, two of the most frequently reported chromosomal alterations in sporadic breast cancer are chromosomes arms 1p and 8p (17, 18). Allelic loss of 1p is observed frequently in breast and colon cancer, neuroblastoma, and melanoma (17). Allelic loss of chromosome arm 8p is commonly seen in several cancers including invasive breast carcinoma with poor prognosis, indicating the presence of one or more TSGs in this region (18). In addition to its occurrence in infiltrating ductal cancer, allelic imbalance of 8p has been observed in ductal carcinoma *in situ* (19, 20), and in atypical ductal hyperplasia (21), suggesting that genes in this region are involved in early aspects of breast cancer progression. In the bcMCF cells, allelic imbalance of chromosome arms 1p and 8p are two of the five observed regions of chromosomal instability associated with a tumorigenic phenotype (Table 2), lending support to the relevance of this cell model to breast cancer in women.

An earlier study of sporadic breast carcinoma used 19 microsatellite makers to determine the frequencies and patterns of allelic loss on chromosome 4 (22). Ninety-one percent of the breast cancers examined showed allelic loss of chromosome 4. The degree of loss ranged from

complete loss of an allele to small sub-regions of loss. Four frequently observed sub-regions were identified at 4p15, 4p16, 4q25-26 and 4q33-34 (22). Two recent studies, using a GeneChip containing 1495 SNPs (23) or comparative genomic hybridization (24), showed that LOH and allelic loss in chromosome 4p and 5q occur more frequently in subtypes of breast cancer characterized as ER-negative. SNP mapping identified regions of chromosome 4p14-4p15 and 5q11-5q35, and associated these changes with a "basal-like" gene expression profile (23). The study using comparative genomic hybridization identified the regions 4p16 and 5q23-5q35, as well as regions of several other chromosomes (24). In the bcMCF cell line, deletions ranging from complete loss of chromosome 4 to seven specific sub-regions in bcMCF clone A (bcMCF1) were identified (Table 2). Of interest, the bcMCF cells also exhibited allelic imbalance at chromosome 5 corresponding to 5q21.1-5q35.3 (Table 2). In contrast to the results of the earlier study (24), we observed an amplification of chromosome 5 in the bcMCF cells, not a deletion. However, an existing region of LOH was identified in all of the cell lines at 5q33.2. One candidate gene, ABLIM3, showing differential expression in the bcMCF cell line, is identified in this region (Table 2).   Of interest, a region of chromosome 5, 5q33-34, was previously identified as a possible *BRCA1* modifier locus, affecting the penetrance of breast cancer due to germline mutations in BRCA1 (25).

That four of the five regions of chromosomal imbalance found in the bcMCF cells have been strongly associated with breast cancer in women supports the relevance of this model. Furthermore, the similarities found at chromosomes 4 and 5 between this ER negative model and the subtypes of cancer in women characterized as ER negative and "basal-like" suggest that this cell model may be of special importance to the study of these cancers with poor prognosis. In this regards, it is possible that the trMCF cells, transformed but not tumorigenic, may represent

the earliest breast cancer phenotype of these types of cancer that has ever been observed. Further

studies of the specific genes in these regions and they relationship to malignant transformation

are underway.

Towards that goal, the identification and prioritization of the candidate oncogenes and

TSGs is an important aspect of this study. By integrating the high resolution 100k SNP mapping

data with the comprehensive gene expression data of the HG-U133_Plus_2 array we were able to

enumerate the ordered expressed gene list corresponding to each region of chromosomal change

(Table 1-2). As presented in the Results section, this integrated approach greatly narrows the list

of candidate gene in a specific region, in most cases by more than 50 percent.

The earliest chromosomal abnormality observed in this model is in the trMCF cells for

chromosome 1p36.21-1p36.12. Several candidate genes are identified for this region (Table 1).

Altered levels of expression and function of EPHA2, an ephrin family receptor tyrosine kinase,

has been associated with the transformation of epithelial cells, including breast, as well as

clinical cases of breast and other cancers (26). Chromosome 1 open reading frame 33, C1orf33,

shows the strongest, statistically significant increase in RNA expression occurring in this region

of LOH and subsequent amplification. However, the function of this gene product is not known.

Proximal to this gene is AKR7A2, an aldo-keto reductase protein previously associated with

colorectal cancer in humans (27). Another gene, NBL1, also known as DAN or NO3, encodes a

neuroblastoma tumor suppressor gene whose expression is decreased in transformed cells (28).

These and many other genes are expressed in this region of expanded amplification found

in bcMCF cells at chromosome 1pter-1p36.12 (Table 2). One gene of interest to breast cancer is

MAD2L2 (or mitotic arrest deficient, yeast homolog-like 2). In yeast, the MAD2L2 homolog,

Rev7p, is found in a protein complex with Rev3p, the catalytic subunit of DNA polymerase zeta.

This complex is known to contribute to error-prone postreplication repair of DNA damage and is associated with chromosomal instability (29). Increased expression of mitotic checkpoint genes has been shown to occur in breast cancer cells with chromosomal instability (30).

The second region of amplification in bcMCF cell occurs in chromosome 5 at 5q21.1-5q35.3. Of great interest is the enhanced expression of LOX, lysyl oxidase, an extracellular matrix remodeling enzyme. The expression of LOX is up-regulated in invasive breast cancer and the activity of LOX has been shown to facilitate cell invasion *in vitro* (31). Through a hydrogen-peroxide-mediated mechanism, LOX has been shown to promote the focal adhesion kinase and Src kinase signaling pathway, leading to enhanced cell migration and invasiveness (32). Another interesting set of genes are those identidfied from ABLIM3 to RARS (Table 2), as these genes correspond to the region of chromosome 5 previously associated with ER negative breast cancer (24), as well as a modifier locus of BRCA1 (25). Expression of ABLIM3 is significantly up-regulated in the bcMCF cells and is the only such gene found a region of existing LOH (Table 2). Three additional genes in this region, G3BP, PTTG1 and HMMR have been linked to breast cancer. G3BP is known to be over-expressed in breast cancer and is believed to play a role in morphological remodeling and adhesion (33). Overexpression of PTTG1 leads to cell transformation that is accompanied by disruption of mitosis and aneuploidy and it has been shown that its expression is elevated in several types of tumors including breast (34). Overexpression of HMMR has been shown to transform murine fibroblasts and its expression is required for the cell transformation effect of H-ras (35).

The final region of amplification in the bcMCF cells occurs at 13q21.31-13q34. Of interest, the expression of KLF5 is significantly lower (-3.3 fold) even though it resides in this

region of chromosomal amplification. KLF5 is a tumor suppressor gene whose expression is

markedly decreased in breast cancer cells (36).

In bcMCF clone A (bcMCF1), shown to be tumorigenic, several discrete regions of

chromosome 4 deletion were observed. The deletion at 4p16.1 is of interest because this region

has been associated with ER negative breast cancer (24). The level of expression of WDR1 is

significantly lower in bcMCF cells (-1,3 fold). Knock-down of WDR1 in several cell lines

resulted in cell flattening and abnormal chromosomal segregation and an increase in the

percentage of multinucleated cells (37). A second gene of interest, located at 4q31.3, is FBXW7;

mutations in this gene lead to aberrant cell cycle regulation of cyclin E, which is often over-

expressed in breast cancer (38) and can cause chromosomal instability (39).

The deletion in bcMCF cells at chromosome 8p23.1-8p11.1 contains 92 expressed genes.

Because allelic imbalance of the 8p arm is common is several cancers, a large number of these

genes have been associated with cancer in some manner. As it is not possible to discuss each of

these genes, we have chosen several with specific interest to breast cancer or cell transformation.

The expression of PDLIM2 is decreased 5-fold in bcMCF cells (Table 2); knockdown of this

gene abrogated adhesion in both MCF-7 cells and the non-transformed MCF-10A cells (40).

KIAA1967, also called DBC2 (deleted in breast cancer) encodes a protein of unknown function

and it has been identified as the most likely candidate tumor suppressor gene associated with

deletion at 8p21 in breast cancer (41). TRIM 35, also called HLS5 (hemopoietic lineage switch

5), has been identified as a candidate TSG for leukemias and solid tumors at 8p21 and enforced

expression of this gene in HeLa cells inhibited cell growth, clonogenicity, and tumorigenicity

(42).   A complex variety of chromosomal 8p alterations have been observed in breast cancer,

including loss of 8p21-ter, amplification of 8p12-8p11, and breaks in the region of 8p21-8p12.

Although only 8p deletion is found in the bcMCF cells, the genes in the region bounded by KIF13B and FNTA correspond to a region of chromosome 8 that is frequently amplified (43). The sub-region bounded by genes LSM1 and ADAM9 correspond to amplicon 2 of a recent report showing a strong association between amplicon 2 and poor breast cancer prognosis (43). The level of expression of SFRP1, secreted frizzled-related protein, is the most dramatic of any of the gene identified in Table 2, showing a 28-fold down-regulation, corresponding to an absent signal in the Affymetrix file. SFRP1 is an extracellular antagonist to Wnt signaling that acts by binding to Wnt proteins. Expression of SFRP1 is lost in more than 80 percent of invasive breast carcinomas, except in the medullary type (44). In a recent study of more than 2000 invasive breast tumors and 56 carcinoma *in situ*, the frequencies of SFRP1 loss were reported as 46 and 43 percent, respectively. These authors concluded that loss of SFRP1 is an early event in breast cancer. Furthermore, they showed that loss of SFRP1 expression in early stage breast tumors was associated with poor prognosis (45).

In summary, we have identified many similarities between the genomic changes occurring during the 17 beta estradiol induced malignant transformation of MCF10F cells and breast cancer in women. The ability to study the function of candidate genes in this model should improve our understanding of breast cancer progression.

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Figure Legends

Figure 1. Schematic of the experimental protocol. The trMCF are MCF10F cells transformed with 70nM E2. The transformation phenotype (colony formation in agar-methocel, ductulogenic capacity, invasiveness capacity) was validated as described before. The bsMCF are invasive trMCF cells selected by growth through matrigel in a Boyden chamber. The bcMCF are clones of the bsMCF cells. The bsMCF and bcMCF cell lines are tumorigenic in SCID mice. The caMCF are cell lines established from the solid tumors of SCID mice.

Figure 2. Chromosome copy number view of the parental MCF10F cell line compared to a normal human reference set, and displayed by CNAT v3.0 using genomic smoothing analysis. In each panel, the upper line shows copy number and the lower line shows the associated p-value. A region of copy number deletion was detected at 1pter (red shading below the line); a region of copy number amplification was detected at 8qter (blue shading above the line).

Figure 3. Concurrent analysis of chromosomal copy number (CN) and loss of heterozygosity (LOH). As described in the Materials and Methods section, dCHIP software was used to examine both copy number and allelic alterations. A) (Pink). Complete genome view of copy number, with darker and lighter areas representing areas of copy number amplification and deletion, respectively. The gray box to the right represents the value range from 0 to 4 copies; the red line represents the normal 2 copy. B) (Yellow/Blue). Complete genome view of LOH (yellow, retention of heterozygosity; white, no information due to lack of SNPs; blue, LOH). The cell lines, MCF10F1-3, trMCF1-3, bsMCF1-3, bcMCF1-3, and caMCF1-3 are as described in Fig. 1. Three independent samples, and three independent cell clones for bcMCF, were analyzed for each cell line.

Figure 4. Higher resolution analysis of chromosome 4. *A*, Chromosome 4 copy number is displayed by CNAT v3.0 using genomic smoothing analysis for each cell line. In each panel, the right line shows copy number and the left line shows the associated p-value. *B*, LOH analysis of chromosome 4 in each cell line. In the bsMCF1 sample and the sub-clone bcMCF1, seven discrete regions of chromosome 4 LOH are identified.

Table 1. Chromosomal Abnormalities in trMCF.

Chromosomal region	Chromosomal position (Mb)		Type of aberration	Genes	Description	Fold-change[a]
	Star	Stop				
1p36.21-1p36.12[b]	14.469	20.912	Amplif.	SPEN[c]	spen homolog, transcriptional regulator (Drosophila)	1.07
				EPHA2[c]	EPH receptor A2	-1.3
				SDHB	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	1.6
				PADI1	peptidyl arginine deiminase, type I	-2.4
				RCC2	regulator of chromosome condensation 2	1.3
				C1orf33	chromosome 1 open reading frame 33	2.9
				AKR7A2[c]	aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)	1.2
				NBL1[c]	neuroblastoma, suppression of tumorigenicity 1	1.1
				CAMK2N1	calcium/calmodulin-dependent protein kinase II inhibitor 1	-2.1

[a]Expression value relative to MCF10F; shaded genes are discussed in text.
[b]LOH exist in this entire region due to existing deletion in MCF10F cells.
[c]Fold-change value was not statistically significant, yet gene is located in region of LOH and has a known relation to cancer.

Table 2. Chromosomal Abnormalities in bcMCF

Chromosomal region	Chromosomal position (Mb)		Type of aberration	Genes	Description	Fold-change[a]
	Star	Stop				
1 pter- 1p36.12[b]	0.328	20.912	Amplific.	NOC2L	nucleolar complex associated 2 homolog (S. cerevisiae)	2.2
				AGRN	agrin	-2.3
				SDF	stromal cell derived factor 4	2.5
				UBE2J2	ubiquitin-conjugating enzyme E2, J2 (UBC6 homolog, yeast)	2.0
				AURKAIP1	aurora kinase A interacting protein 1	2.2
				GNB1	guanine nucleotide binding protein (G protein), beta polypeptide 1	1.3
				PEX10	peroxisome biogenesis factor 10	1.6
				LRRC47	leucine rich repeat containing 47	2.4
				RPI-286D6.4	Glycine-, glutamate-, thienylcyclohexylpiperidine-binding protein	-3.3
				KCNAB2	potassium voltage-gated channel, shaker-related subfamily, beta member 2	6.4
				RPL22	ribosomal protein L22	1.5
				ICMT[c]	isoprenylcysteine carboxyl methyltransferase	1.4
				ACOT7	acyl-CoA thioesterase 7	4.4
				NOL9	nucleolar protein 9	2.3
				PHF13	PHD finger protein 13	1.3
				VAMP3	vesicle-associated membrane protein 3 (cellubrevin)	1.4
				PARK7	Parkinson disease (autosomal recessive, early onset) 7	1.6
				ERRFI1	ERBB receptor feedback inhibitor 1	2.6
				RERE	arginine-glutamic acid dipeptide (RE) repeats	1.8
				ENO1	enolase 1, (alpha)	1.5
				CLSTN1	calsyntenin 1	-1.7
				UBE4B[c]	ubiquitination factor E4B (UFD2 homolog, yeast)	1.1
				DFFA[c]	DNA fragmentation factor, 45kDa, alpha polypeptide	1.8
				SRM	spermidine synthase	3.9
				FRAP1[c]	FK506 binding protein 12-rapamycin associated protein 1	1.3
				MAD2L2	MAD2 mitotic arrest deficient-like 2 (yeast)	3.1
				KIAA2013	KIAA2013	2.2
				PLOD1	procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1	3.1
				MFN1	mitofusin 2	1.7
				PRDM2[c]	PR domain containing 2, with ZNF domain	1.3
				EFHD2	EF-hand domain family, member D2	3.2
				SPEN[c]	spen homolog, transcriptional regulator (Drosophila)	1.4
				EPHA2[c]	EPH receptor A2	-1.3
				ATP13A2	ATPase type 13A2	1.5
				SDHB	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	2.8
				RCC2	regulator of chromosome condensation 2	1.4
				KIAA0090	KIAA0090	4.0
				C1orf33	chromosome 1 open reading frame 33	4.6

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			AKR7A2[c]	aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)	1.7
			CAPZB	capping protein (actin filament) muscle Z-line, beta	1.6
			C1orf51	chromosome 1 open reading frame 151	1.8
			NBL1[c]	neuroblastoma, suppression of tumorigenicity 1	1.3
			CAMK2N1	calcium/calmodulin-dependent protein kinase II inhibitor 1	-2.4
			DDOST	dolichyl-diphosphooligosaccharide-protein glycosyltransferase	2.0
5q21.1-5q35.3[d]	101.142 179.773	Amplific.	LOX	lysyl oxidase	2.4
			ALDH7A1	aldehyde dehydrogenase 7 family, member A1	2.1
			HINT1	histidine triad nucleotide binding protein 1	1.4
			PDLIM4	PDZ and LIM domain 4	1.5
			APXL2	Apical protein 2 (est)	1.9
			HSPA4	heat shock 70kDa	2.2
			CAMLG	calcium modulating ligand	1.5
			H2AFY	H2A histone family, member Y	1.2
			HNRPA0	heterogeneous nuclear ribonucleoprotein A0	1.4
			KIF20A	kinesin family member 20A	2.4
			HSPA9B	heat shock 70kDa protein 9B (mortalin-2)	1.8
			PAIP2	poly(A) binding protein interacting protein 2	1.7
			IK	IK cytokine, down-regulator of HLA II	1.9
			HARS	histidyl-tRNA synthetase	1.8
			ZMAT2	zinc finger, matrin type 2	1.6
			LARS	leucyl-tRNA synthetase	3.1
			ABLIM3[e]	actin binding LIM protein family, member 3	1.9
			RBM22	RNA binding motif protein 22	1.9
			MST150	MSTP150 (est)	4.4
			ANXA6	annexin A6	3.4
			G3BP	Ras-GTPase-activating protein SH3-domain-binding protein	1.9
			PTTG1	pituitary tumor-transforming 1	2.2
			HMMR	hyaluronan-mediated motility receptor (RHAMM)	2.4
			MAT2B	methionine adenosyltransferase II, beta	1.8
			RARS	arginyl-tRNA synthetase	1.5
			FLJ20364	Hypothetical protein FLJ20364 (est)	2.9
			NPM1	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	1.2
			STK10	serine/threonine kinase 10	1.4
			DUSP1	dual specificity phosphatase 1	3.1
			RPL26L1	ribosomal protein L26-like 1	1.5
			THOC3	THO complex 3	1.5
			HSPC111	Hypothetical protein HSPC111 (est)	1.9
			HIGD2A	HIG1 domain family, member 2A	1.4
			RAP80	Receptor associated protein 80 (est)	1.8
			LMAN2	lectin, mannose-binding 2	1.4
			NOLA2	nucleolar protein family A, member 2 (H/ACA small nucleolar RNPs)	1.5
			HNRPAB	heterogeneous nuclear ribonucleoprotein A/B	1.3
			CANX	calnexin	1.5
			SQSTM1	sequestosome 1	2.1
			GFPT2	glutamine-fructose-6-phosphate	18.0

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					transaminase 2		
13q21.31-13q34[f]	62.699	112.940	Amplific.	PCDH9	protocadherin 9		2.1
				KLF5	Kruppel-like factor 5 (intestinal)		-3.3
				KCTD12	potassium channel tetramerisation domain containing 12		9.8
				ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4		1.9
				RANBP5	RAN binding protein 5		2.0
				TPP2	tripeptidyl peptidase II		3.0
				BIVM	basic, immunoglobulin-like variable motif containing		2.5
				LIG4	ligase IV, DNA, ATP-dependent		-2.5
				FLJ12118	Hypothetical protein FLJ12118 (est)		1.9
				ING1	inhibitor of growth family, member 1		1.4
				ATP11A	ATPase, Class VI, type 11A		-5.0
				FLJ11305	Hypothetical protein FLJ11305 (est)		2.1
4p16.1-4p16.1[g]	8.657	10.435	Deletion	DRD5	dopamine receptor D5		-1.0
				WDR1	WD repeat domain 1		-1.3
4q13.1-4q13.2	65.333	67.666	Deletion	None expressed			
4q21.21-4q21.21	79.795	82.427	Deletion	ANAX3	annexin A3		-9.0
				BMP2K	BMP2 inducible kinase		-2.2
				PAQR3	progestin and adipoQ receptor family member III		-2.1
				ANTXR2	anthrax toxin receptor 2		1.58
4q25-4q25	108.324	109.815		PAPSS1	3'-phosphoadenosine 5'-phosphosulfate synthase 1		-2.0
				MGC26963	Hypothetical protein MGC26963 (est)		-3.6
				CYP2U1	cytochrome P450, family 2, subfamily U, polypeptide 1		-1.1
				HADHSC	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain		-2.2
4q28.3-4q28.3	134.451	135.178	Deletion	None expressed			
4q31.23-4q32.1	150.275	156.349	Deletion	LRBA	LPS-responsive vesicle trafficking, beach and anchor containing		-4.1
				RPS3A	ribosomal protein S3A		-1.1
				SH3D19	SH3 domain protein D19 (est)		-2.5
				FBXW7[c]	F-box and WD-40 domain protein 7 (archipelago homolog, Drosophila)		-1.2
				FlJ32028	Hypothetical protein FLJ32028 (est)		-6.9
				ARFIP1	ADP-ribosylation factor interacting protein 1 (arfaptin 1)		-2.0
				KIAA1727	KIAA1727 protein (est)		-2.1
				TRIM2	tripartite motif-containing 2		-5.6
				GAJ	GAJ protein (est)		-1.2
				KIAA0922[h]	KIAA0922 protein (est)		-1.7
4q33-4q33	171.612	172.551	Deletion	Non expressed			
8p23.1-8p11.1[i]	12.030	43.322	Deletion	FAM86B1[h]	family with sequence similarity 86, member B1		1.4
				TUSC3	tumor suppressor candidate 3		-1.9

			ZDHHC2	zinc finger, DHHC-type containing 2	-2.4
			CNOT7[c]	CCR4-NOT transcription complex, subunit 7	-1.9
			VPS37A	vacuolar protein sorting 37A (yeast)	-2.5
			MTUS1	mitochondrial tumor suppressor 1	-4.4
			FGL1[c]	fibrinogen-like 1	-1.0
			PCM1[h]	pericentriolar material 1	-1.3
			ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	-5.9
			NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	-6.4
			PSD3[c]	pleckstrin and Sec7 domain containing 3	-2.8
			SH2D4A[h]	SH2 domain containing 4A	-1.6
			ChGn	Chondroitin beta1,4 N-acetylgalactosaminyltransferase (est)	-11.0
			C8orf35[h]	chromosome 8 open reading frame 35	-1.6
			LPL	lipoprotein lipase	-1.7
			ATP6V1B2[h]	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B2	-1.4
			XPO7[h]	exportin 7	1.2
			EPB49[c]	erythrocyte membrane protein band 4.9	1.9
			RAI16	retinoic acid induced 16	-3.2
			REEP4[h]	receptor accessory protein 4	-1.3
			BMP1[h]	bone morphogenetic protein 1	-3.5
			POLR3D[h]	polymerase (RNA) III (DNA directed) polypeptide D, 44kDa	-1.1
			SLC39A14[h]	solute carrier family 39 (zinc transporter), member 14	-1.2
			PPP3CC[h]	protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform (calcineurin A gamma)	1.2
			SORBS3[h]	sorbin and SH3 domain containing 3	-1.5
			PDLIM2	PDZ and LIM domain 2 (mystique)	-5.0
			KIAA1967[c]	KIAA1967	-1.3
			BIN3[h]	bridging integrator 3	-1.5
			TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	-3.5
			TNFRSF10C[h]	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	-1.3
			TNFRSF10D	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	-2.5
			CHMP7	CHMP family, member 7	-1.7
			LOXL2	lysyl oxidase-like 2	-4.7
			ENTPD4	ectonucleoside triphosphate diphosphohydrolase 4	-4.8
			SLC25A37[h]	solute carrier family 25, member 37	-1.2
			NKX3-1[c]	NK3 transcription factor related, locus 1	-1.6
			STC1	stanniocalcin 1	-3.2
			DOCK5	dedicator of cytokinesis 5	-2.4
			KCTD9[h]	potassium channel tetramerisation domain containing 9	-2.1
			CDCA2[h]	cell division cycle associated 2	-1.1
			PPP2R2A[h]	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform	-2.0
			BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	-2.3
			DPYSL2[h]	dihydropyrimidinase-like 2	1.1
			TRIM35	tripartite motif-containing 35	-1.4

				PTK2B[c]	PTK2B	1.6
				CCDC25[h]	coiled-coil domain containing 25	-1.5
				PBK[h]	PDZ binding kinase	-1.3
				ELP3[h]	elongation protein 3 homolog (S. cerevisiae)	-1.3
				ZNF395[h]	zinc finger protein 395	1.1
				FBXO16	F-box protein 16	-1.8
				FZD3	frizzled homolog 3 (Drosophila)	-1.7
				RC74[h]	Integrator complex subunit 9 (est)	-1.4
				FLJ21616[h]	Hypothetical protein FLJ21616 (est)	-1.3
				KIF13B	kinesin family member 13B	-11.0
				DUSP4	dual specificity phosphatase 4	-2.4
				TMEM66[h]	transmembrane protein 66	-2.2
				LEPROTL1	leptin receptor overlapping transcript-like 1	-1.6
				DCTN6	dynactin 6	-2.6
				RBPMS	RNA binding protein with multiple splicing	-3.2
				GTF2E2[h]	general transcription factor IIE, polypeptide 2, beta 34kDa	-1.7
				GSR[h]	glutathione reductase	-1.3
				UBXD6[h]	UBX domain containing 6	-1.2
				PPP2CB[c]	protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	-1.6
				RBM13[h]	RNA binding motif protein 13	-1.2
				C8orf41[f]	chromosome 8 open reading frame 41	-1.2
				SPFH2	SPFH domain family, member 2	-2.6
				PROSC[h]	proline synthetase co-transcribed homolog (bacterial)	1.0
				BRF2[c]	BRF2, subunit of RNA polymerase III transcription initiation factor, BRF1-like	1.0
				RAB11FIP1[h]	RAB11 family interacting protein 1 (class I)	-1.1
				EIF4EBP1[c]	eukaryotic translation initiation factor 4E binding protein 1	-1.6
				ASH2L[h]	ash2 (absent, small, or homeotic)-like (Drosophila)	-1.8
				LSM1	LSM1 homolog, U6 small nuclear RNA associated (S. cerevisiae)	-2.2
				BAG4[h]	BCL2-associated athanogene 4	-1.9
				DDHD2	DDHD domain containing 2	-3.0
				PPAPDC1B	phosphatidic acid phosphatase type 2 domain containing 1B	-2.6
				WHSC1L1	Wolf-Hirschhorn syndrome candidate 1-like 1	-2.2
				LETM2	leucine zipper-EF-hand containing transmembrane protein 2	-1.6
				FGFR1[c]	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	3.2
				TACC1	transforming, acidic coiled-coil containing protein 1	-2.1
				TM2D2	TM2 domain containing 2	-2.4
				ADAM9[c]	ADAM metallopeptidase domain 9 (meltrin gamma)	-2.9
				SFRP1	secreted frizzled-related protein 1	-28.0
				GOLGA7[h]	golgi autoantigen, golgin subfamily a, 7	-2.3
				MYST3[c]	MYST histone acetyltransferase (monocytic leukemia) 3	-1.4
				AP3M2[h]	adaptor-related protein complex 3, mu 2 subunit	-1.3
				PLAT[c]	plasminogen activator, tissue	1.4
				IKBKB[c]	inhibitor of kappa light polypeptide gene	-1.8

				enhancer in B-cells, kinase beta	
			POLB	polymerase (DNA directed), beta	-1.9
			VDAC3[h]	voltage-dependent anion channel 3	-1.5
			C8orf40[h]	chromosome 8 open reading frame 40	-1.9
			RNF170	ring finger protein 170	-2.6
			FNTA[h]	farnesyltransferase, CAAX box, alpha	-1.7

[a]Expression value relative to MCF10F, shaded genes are discussed in text.

[b]LOH exist in this entire region due to existing deletion in MCF10F cells.

[c]Fold-change value was not statistically significant, yet the gene is located in a region of LOH and has a known relation to cancer.

[d]In all cell lines, a region of LOH at 5q33.1 is consistent with a mechanism of recombination with gene conversion and subsequent amplification.

[e]Fold-change value for ABLIM3 was statistically significant, showing increased expression in the 5q33.1 region of LOH and amplification.

[f]This region of chromosome 13 contains two sub-regions of existing LOH. Only the one region at 13q31.1 contains three expressed genes. None of these genes had a fold-change value that was statistically significant.

[g]The seven regions of deletion with LOH identified in bcMCF clone A (tumorigenic) are presented. Three regions contained no expressed genes.

[h]Fold-change value was not statistically significant, and the gene has not been associated with cancer.

[i]Expressed genes located in this region of deletion and LOH of chromosome 8 are identified.
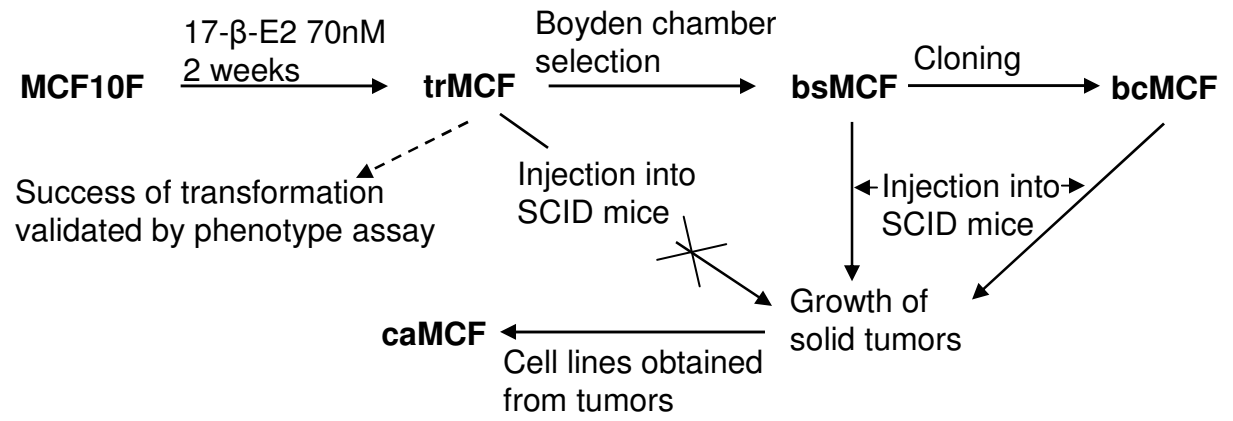
Fig 1

MCF10F —17-β-E2 70nM 2 weeks→ trMCF —Boyden chamber selection→ bsMCF —Cloning→ bcMCF

trMCF ⇢ Success of transformation validated by phenotype assay

Injection into SCID mice

Injection into SCID mice

Growth of solid tumors

caMCF ← Cell lines obtained from tumors
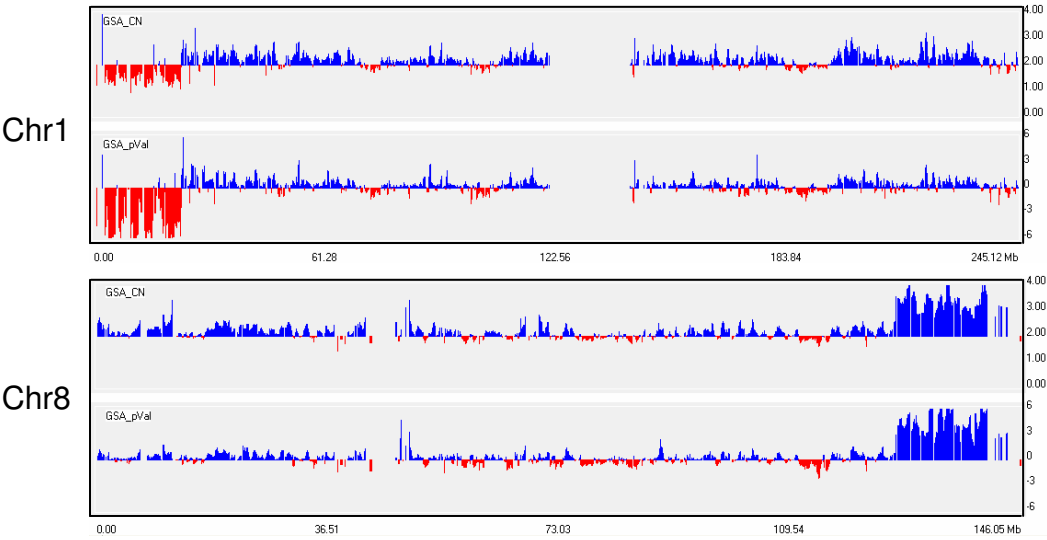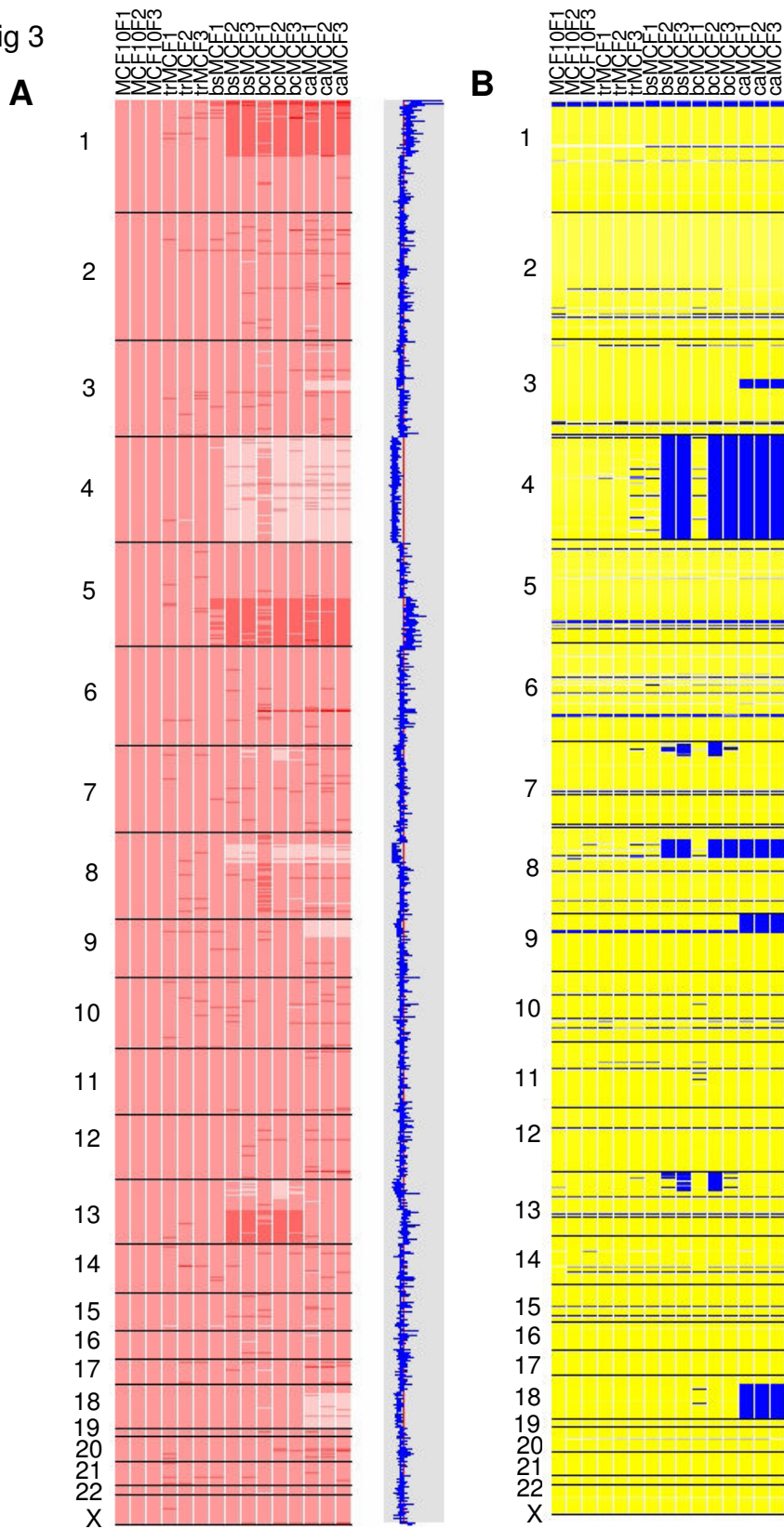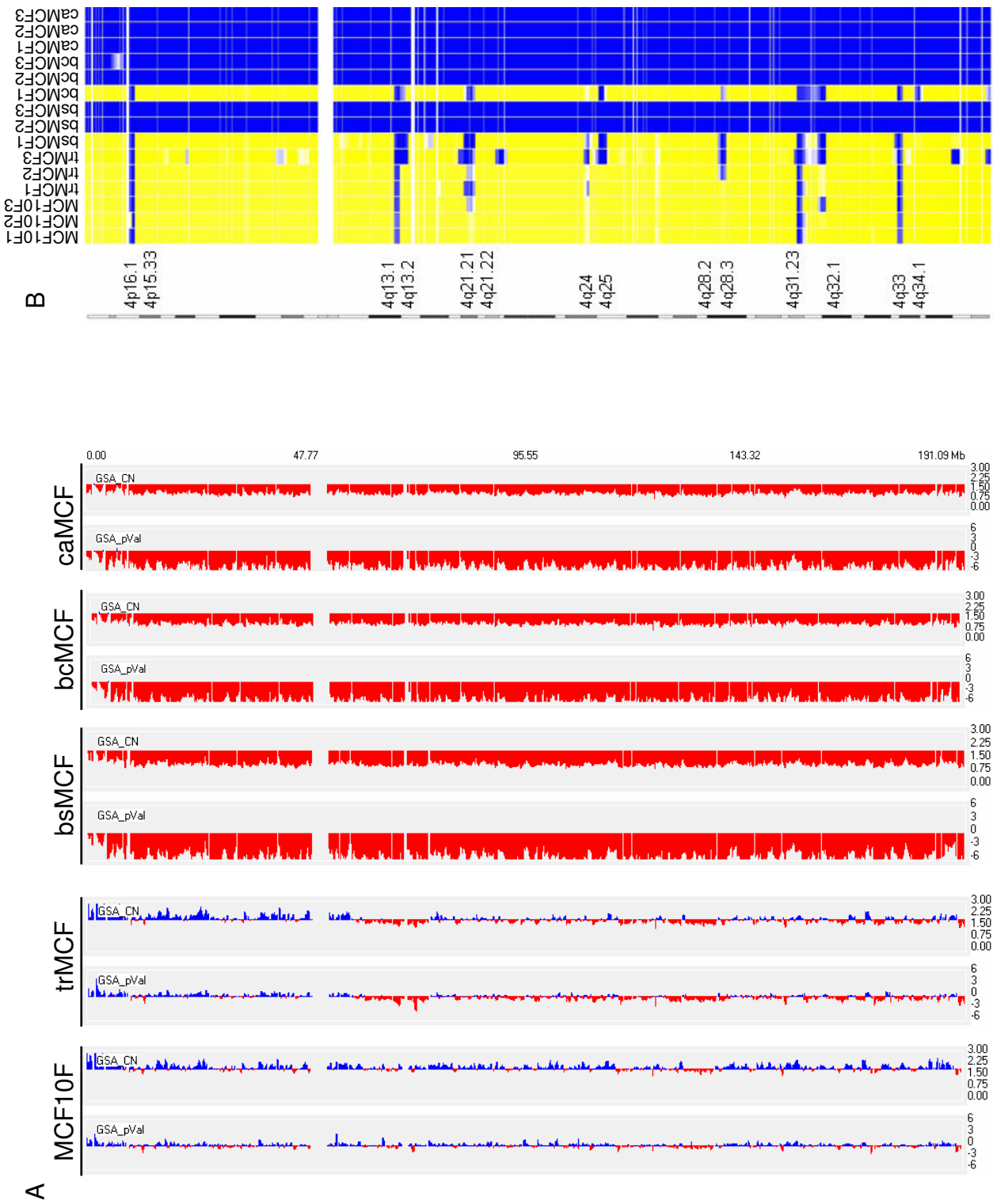
Fig 2

Fig 3

Fig 4

ANALYTICAL CORE – ROGAN

A.   **Introduction**

The Analytical Core provides consistent analytical power to the research projects so that estrogen metabolites, estrogen conjugates and estrogen-DNA adducts can be identified and quantified with the most sensitivity and reliability. The HPLC with multi-channel electrochemical detection enables detection of 31 metabolites and conjugates at the picomole level in one run. A second set-up is used exclusively to analyze the 6 depurinating catechol estrogen-DNA adducts, which need a different elution buffer to achieve separation.   Our newer ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) instrumentation has increased the sensitivity of our analyses to the femtomole level and provides confirmation of structures.

A.   **Body**

B-i.   **Methods and Procedures**

The Analytical Core uses both HPLC with electrochemical detection and UPLC/MS/MS to analyze estrogen metabolites, conjugates and depurinating DNA adducts. A variety of types of samples are analyzed. These include *in vitro* reaction mixtures, human and animal tissue extracts, cell culture medium extracts, and human fluids, such as urine, serum and nipple aspirate fluid. Depending on the sample, preparation for analysis is as simple as filtration through a 5,000-molecular weight filter, or as complex as grinding minced tissue in liquid nitrogen, incubation with glucuronidase/sulfatase, passage through a Sep-Pak column to extract desired analytes, and filtration through a 5,000-molecular weight filter.

B-ii.   **Results**

In the past year our new Acquity UPLC (Waters, Inc), which is coupled with a MicroMass QuattroMicro tandem mass spectrometer has enabled us to reduce the time required for each sample from 1 h to 15 min. More importantly, we can now separate and analyze 36 estrogen metabolites, GSH conjugates [and their breakdown products containing cysteine (Cys) and *N*-acetylcysteine (NAcCys)] and depurinating DNA adducts by UPLC/MS/MS, including 2-OHE_1 and 4-OHE_1.

In the past year we analyzed human samples, including nipple aspirate fluid from women with and without breast cancer, urine from women with and without breast cancer, and serum from women without breast cancer (results described in the report for Specific Aim 1).

We also analyzed medium from MCF-10F cells incubated with E_2 in Specific Aim 2. We detected estrogen metabolites, estrogen conjugates and depurinating estrogen-DNA adducts. We detected for the first time the adducts 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua formed by MCF-10F cells. These results are described in a recently submitted publication [1].

We analyzed mammary tissue and serum from ERKO/Wnt-1 mice and aromatase-transfected mice from Dr. Santen's laboratory (Specific Aim 4). We detected estrogen metabolites, conjugates and depurinating DNA adducts in the mammary tissue and serum from ovariectomized ERKO/Wnt-1 mice in a dose-related manner, depending on the dose of E_2 implanted into the mice. When the aromatase-transfected mice were given testosterone, we detected estrogen metabolites, conjugates and depurinating DNA adducts. When, however, the

mice were also treated with letrozole, an aromatase inhibitor, we could not reliably detect any estrogen products, as expected. These results are described more completely in Specific Aim 4.

B-iii.   Proposed Research for the Next Year
1.   Analyze nipple aspirate fluid samples from women with and without breast cancer (Specific Aim 1).

2.   Analyze urine and serum samples from women with and without breast cancer (Specific Aim 1).

3.   Analyze media from MCF-10F cells at various stages of transformation after treatment with E_2 or 4-OHE$_2$ (Specific Aim 2).

4.   Analyze media from BB2 rat embryonic cells treated with 4-OHE$_2$ (Specific Aim 3).

5.   Continue to analyze mammary tissue from ERKO/Wnt-1 mice and aromatase-transfected mice treated in various ways (Specific Aim 4).

C.   Key Research Accomplishments
We have established a procedure with the Acquity UPLC/QuattroMicro MS/MS to analyze 36 estrogen metabolites, GSH conjugates and depurinating DNA adducts.

We have successfully analyzed human nipple aspirate fluid and detected estrogen-DNA adducts in samples from women with breast cancer, but not in samples from women without breast cancer.

We have successfully analyzed human urine samples from women with and without breast cancer and found that the level of DNA adducts in urine from women with breast cancer is significantly higher than the level of adducts in urine from healthy control women.

We have successfully analyzed human serum samples from healthy control women and found baseline levels of estrogen metabolites, conjugates and depurinating DNA adducts.

D.   Reportable Outcomes
Cavalieri, E., Chakravarti, D., Guttenplan, J., Hart, E., Ingle, J., Jankowiak, R., Muti, P., Rogan, E., Russo, J., Santen, R. and Sutter, T. Catechol estrogen quinones as initiators of breast and other human cancers. Implications for biomarkers of susceptibility and cancer prevention. BBA-Reviews on Cancer, in press, 2006.

Saeed, M., Rogan, E., Sheriff, F., Fernandez, S., Russo, J. and Cavalieri, E. Formation of depurinating N3adenine and N7guanine adducts by MCF-10F cells cultured in the presence of 4-hydroxyestradiol. Int. J. Cancer, submitted, 2006.

Cavalieri, E.L. and Rogan, E.G. The etiology of breast cancer. Prevention is now the solution. Era of Hope, Department of Defense Breast Cancer Research Meeting, Philadelphia, PA, June 8-11, 2005

Rogan, E.G. and Cavalieri, E.L. The role of estrogen metabolism in the initiation of human breast cancer. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Philadelphia, Pennsylvania, June 8-11, 2005.

Lu, F., Cavalieri, E.L. and Rogan, E.G. TCDD-induced expression of cytochrome P450 enzymes and formation of estrogen metabolites in human breast epithelial MCF-10F cells. Proc. Amer. Assoc. Cancer Res., **47**, 445, Washington, DC, April 1-5, 2006.

Saeed, M., Rogan, Ed., Cavalieri, E., Sheriff, F., Fernandez, S. and Russo, J. Formation of the DNA depurinating N3Ade and N7Gua adducts of 4-hydroxyestradiol by MCF-10F cells cultured with the carcinogenic estrogen metabolite 4-hydroxyestradiol. Proc. Amer. Assoc. Cancer Res., **47**, 447, Washington, DC, April 1-5, 2006.

E.   Conclusions
The Analytical Core has successfully implemented UPLC/MS/MS to detect estrogen metabolites, GSH conjugates and depurinating DNA adducts in human nipple aspirate fluid and urine from women with and without breast cancer. The depurinating DNA adducts are present at higher levels in women with breast cancer, compared to healthy control women. We are also analyzing estrogen metabolites, GSH conjugates and depurinating DNA adducts in human serum from healthy control women and will compare the levels with those in serum from women with breast cancer.

The Analytical Core is interacting with the other investigators in the Breast Cancer Center of Excellence to analyze a variety of samples for estrogen metabolites, estrogen GSH conjugates and depurinating estrogen-DNA adducts.

F.   References
1.   Saeed, M., Rogan, E., Sheriff, F., Fernandez, S., Russo, J. and Cavalieri, E. Formation of depurinating N3adenine and N7guanine adducts by MCF-10F cells cultured in the presence of 4-hydroxyestradiol. Int. J. Cancer, submitted, 2006.

ADVOCACY CORE - HART

Web-based model reference tool
        We were unsuccessful in interesting TraCorp in developing the reference tool; however, we were put in touch with experts at the Library of Medicine through our advisor, Dr. David Longfellow, who felt they would be interested in our project. Indeed, they were interested and asked that a concise memo be sent detailing our idea and the parameters. They will consider the project, as it dovetails with a similar project they are working on. (See memo) In discussions with the National Library of Medicine, the estimate to develop the tool was approximately $100,000. They will get back to us in the next several months, and we will see if we can work collaboratively or whether our idea and the NLM's can be developed in concert under the auspices of the NLM.

Consumer Guide to Involvement in Basic Research
        The consumer guide is finished and is being circulated to other members of the Breast Cancer Center of Excellence**.** It has been reviewed by a select group of consumer advocates and once the BCCOE members have had the opportunity to review the Guide, it will be disseminated to the advocacy community via the Internet.

Article for publication based on current research

The article is in process and comes at an exciting time in our research as we are now looking at an important biomarker that has been discovered by our group.

One of our members, Ms. Elizabeth "Betsy" Mullen has withdrawn; another of our members is dealing with a recurrence of breast cancer, and the third is dealing with a family member who has been diagnosed with colon cancer metastasized to the liver. However, we continue to be very excited about the direction of our research and participate in all meetings and teleconferences.

Memo

To: Dr. Jack Snyder

        National Library of Medicine

From: Elizabeth A. Hart

Date: 7/28/2006

Re: Research Tool

Research Tool:

Designed For: Researchers, Advocates, Lay Public

Purpose: Provide ability to read scientific articles and published papers in context with scientific word definitions available in a "pop-up" environment as computer cursor scrolls over the word.

> Currently, if a researcher, advocate, or lay person is reading a scientific paper or published article, he/she must leave the article, go to a glossary, look up the word, get out of the glossary and return to the article with the definition in mind or written down to continue reading. Not ideal and unnecessary in today's high tech environment.

Rationale for Developing:

> Easy reading in context of publication

> Raises level of scientific understanding, particularly in public domain. Critical to increased public support of research.

> Facilitates the understanding of unfamiliar research to scientists who are involved in other areas of study

> Facilitates participation of advocates in the scientific milieu resulting in increased advocacy in local, state, and federal environments with increased research dollars and interest in research careers

> Utilizes current technology to simplify access to information in the public domain.

1

Proposed solution:

Medical journal articles or scientific articles appear as web pages in a normal web browser window. Before the web server sends the patg, it will automatically run an application that adds invisible hyperlinks to each word it matches to a medical term in its dictionary. While reading the jounal, a user can roll the mouse over the dictionary words and automatically op up the definition. (Research conversation with TraCORP)

Additionally,

One must have the ability to add definitions to the dictionary as new concepts and words are developed in the course of research

Download easily to the individual computer user

Reside in the public domain and readily available to those who want to use it.

From A Consumer Advocates' Perspective

By: Elizabeth A. Hart
        President & CEO
        HART INTERNATIONAL
        Partnerships Advancing Women's Health Research

As a member of the National Cancer Institute's Consumer Advocates in Research and Related Activities (CARRA), I have had the pleasure of serving in several venues including: Special Emphasis Panels, Peer Review Panels, both basic and clinical, and an NCI Special Review Committee for NSABP along with being appointed to the first National Cancer Policy Board. I have testified before the Board of Scientific Counselors, Congressional Committees, President's Cancer Panel, and am currently a member of the Cancer Cube, an NCI supported focus group on estrogen carcinogenesis. Why? I am the daughter of a mother diagnosed and now deceased with breast cancer, a sister who died of breast cancer, a brother who died of throat and lung cancer, another brother who has survived breast and kidney cancer, and a father who has metastatic prostate cancer. Research is vitally important to me and to my family and friends, as is the case with a number of advocates who serve as members of CARRA. Having lived the cancer experience in my family, I am profoundly committed to eradicating this disease and am deeply grateful to the first Director of CARRA, Eleanor Nealon's vision for involving advocates in the research process at NCI. She had a personal understanding of the value advocates could bring to the table, and paved the way for participation in a variety of activities. She had a particular gift in bridging the gap among advocates, the scientific community and government agencies, and we all owe her a debt of gratitude.

My service on peer review panels, both clinical and basic, has been extremely rewarding. I love science, have a hearty understanding of biology, anatomy, physiology and chemistry and feel that I am at times privy to cutting edge ideas that will contribute to the ultimate eradication of cancer. That gives me great hope! The discussions regarding a specific grant application are generally animated with committee members providing their respective insights into a particular application. In terms of basic science applications, one looks for the next piece of the puzzle that will contribute to the overall understanding of the cancer process in the body and particularly the cell. It is important that the science within the application build upon the body of knowledge already published and that the hypothesis will answer an as yet unanswered question in the initiation and proliferation of cancer. If one can tract the process of initiation and proliferation, then one theoretically and actually can find a way to prevent it. I am passionate about the necessity to fund basic research, as it is the means to the ultimate end of cancer. However, in the meantime, the necessity of interrupting the process of cancer must be addressed in clinical trials with new advancements in basic and clinical research being translated to the bedside as expeditiously as possible. Advocates play a major role in articulating the urgency of new treatments, their practicality, safety, compliance and general acceptance. Advocates put a human face on cancer research.

They have lived the cancer experience. It is upon them that the research is ultimately tested, and who better to articulate their perspective? It is a unique opportunity for an advocate to impact the course of research in a positive way. It means that one's personal agenda and that of the organization that one represents takes back seat to the science. Does the hypothesis make sense? Will it add a piece to the puzzle? Is the research plan sound? Does the research build upon established work? These are just a few of the questions I ask as I review proposals. I look at each review experience as one to add to my scientific knowledge. If one approaches each peer review experience with a focus firmly on the science being presented, the experience will be a positive one both for you and for the scientists on the committee.

One of my more challenging experiences was as a member of an NCI Special Review Committee for NSABP. This special review involved a thorough evaluation of NSABP, its many protocols, its statistical group, its participating members, structure, oversight, data safety and monitoring committee, patient advisory board, and many other aspects of a cooperative clinical trial group. There were multiple presentations, much discussion and input from those involved in the group. There were critical questions to be answered from this august group with the ultimate goal of recommending continued funding. It was imperative to make certain that all elements important in conducting clinical trials be in place and that resulting data be above reproach. I am happy to state that this important group among others passed and continues to provide the venue for outstanding clinical research, as do many other cooperative groups funded by the National Cancer Institute. Clinical research is so very important and advocates can play a very significant role in encouraging the public to participate in a clinical trial. NSABP has a wonderful Patient Advisory Committee of loyal participants who work in their respective communities to encourage participation. This is of enormous value in completing accrual for a specific trial in a short period of time and ascertaining whether a particular treatment will be helpful.

The National Cancer Policy Board provided another opportunity for me to make a contribution in the effort to eradicate cancer. I was appointed by the National Academy of Science's Institute of Medicine to the newly formed Board in 1997. The board's mandate was to "confront obstacles and address issues in cancer prevention, control, diagnosis and treatment. It was to examine implications of ongoing research and new technologies, of issues in prevention and care delivery, and of problems 'faced in the nation's battle against cancer', and render advice and recommendations." (JNCI News, Vol.89.No.5. March 5, 1997). One of the first issues the Board addressed was smoking cessation, particularly teenage smoking. The Board reviewed previous work that had been accomplished and worked toward a plan that would impact the young person's initiation into the smoking experience. What intervention methods would work to prevent initiation of smoking? It was an important piece of work with a resulting white paper that was sent to the White House. Actions following the white paper had a significant impact upon the tobacco companies as well as initiating the launch of a smoking cessation campaign aimed at teens.

Another significant cancer issue that the Board addressed was the status of cancer treatment in America. This was a major undertaking requiring expert testimony from a variety of individuals as well as commissioned papers to cover specific aspects requiring

a compilation of data from several different sources. It required a tremendous amount of reading from members of the committee and hours of research on the National Academy of Science's staff. The end result was a publication from the committee *"Ensuring Quality Cancer Care, published* by the National Academy Press in 1999. It was an eye opening report revealing both strengths and weaknesses in the delivery of healthcare to those diagnosed with cancer. Ten significant recommendations were made to improve healthcare treatment and delivery to cancer patients. This particular piece of work was most meaningful to all members of the board and one we all took great pride in completing. Obviously, it led to further efforts by the board, and subsequent boards.

The National Cancer Policy Board was significant in that it was a very diverse group brought together to lend their particular expertise and focus on issues traversing the gamut of the cancer experience. The mandate from the Institute of Medicine was taken very seriously and each member of the board gave above and beyond in an effort to "make a supreme difference." It was a joy to serve with my colleagues on this board as well as the other venues I have been privileged to serve. I firmly believe that it is by working together - advocates, scientific community, government agencies, and private sector - that cancer can be eradicated. We cannot do less. Eleanor knew that. Thank you, Eleanor, and thank you to all those who have given their lives in the hope of eradicating cancer.

PARTNERS IN RESEARCH
ADVOCATES & SCIENTISTS

PARTNERS IN RESEARCH

Advocates & Scientists

Advocate's Guide

Elizabeth A. Hart – HART INTERNATIONAL

PC*r*nerships Advancing Women's Health Research.

Margaret Borwhat – Women's Cancer Advocacy Network (WCAN)

TABLE OF CONTENTS

Introduction

As members of the advocacy community, we are passionately committed to advancing breast cancer research in partnership with scientists in the field. We each have a vital role in eradicating breast cancer and one day, preventing the disease. Scientists have the education in the basic sciences and laboratory techniques, which enable them to conduct basic, translational and clinical research. Advocates, survivors and family members, on the other hand, are the end recipients of the research and the human laboratory to confirm whether the disease hypothesis was indeed correct and the proposed prevention or treatment effective. The two roles are complementary, each informing the other and the focus of this guide to aid advocates who are interested in participation in research activities, specifically basic sciences, to find the avenue that best suits their particular interests. It is not meant to be an exhaustive guide, but a guide to spur the advocate to become involved and the scientist to seek that involvement if he or she has not already done so.

In the age of limited resources to fund research, it is vital for the layperson to understand the necessity for basic scientific research and to be willing to fund such research. The more the lay person understands, the better the chance of increasing funding levels and sources to continue to fight breast cancer and one day prevent it. It is our hope that this brief guide will induce interest among the advocacy community and entice advocates who are reticent to become involved in basic scientific research to take the plunge. The rewards are enormous for the advocate, the scientists and research in general.

Chapter 1

Requisites of Participation

Involvement in basic scientific research for the advocate generally occurs when one has had an experience with cancer at close range, either personally, a family member, or a close friend. The experience is often overwhelming, frightening, sad, frustrating, helpless and hopeful, to name but a few of the emotions that one traverses during the cancer process. Over 136 cancer organizations are listed in the Office of Liaison Activities at the National Cancer Institute alone, helping people in their respective communities find access to care, support in living out the cancer experience, raising money for research, sitting on local, regional, national, and international committees that address research, policy, survivorship, quality of life, cultural competency, prevention, detection, risk and many other important issues.

If one's interest is in participating in basic research – often referred to as bench research – it helps to have some rudimentary knowledge of biology, chemistry, anatomy and physiology. Do not worry if you don't have an understanding of all of these subjects, one will suffice to begin with. The key issue is exposure to a level of science so that you can understand some basic scientific concepts. A strong foundation in science is the goal. If you do not have these requisites in your educational background, invade the local library and READ, READ, READ. Better yet, audit some classes. Ask questions about EVERYTHING you do not understand. The more in-depth exposure you have to scientific concepts, the greater will be your understanding. It does not matter if you don't understand everything that is between the pages on a first read. However, THE MORE YOU READ, THE GREATER YOUR UNDERSTANDING! Take notes; look up words you don't understand. Attend special lectures open to the public at local cancer institutes or community cancer centers, and develop a relationship with a laboratory scientist whom you respect and of whom you may ask questions. Attend scientific meetings and lectures if you are able to do so. Most medical schools have ongoing CME (continuing medical education) conferences for basic, translational, and clinical researchers, treating physicians, nurses and other medical professionals. Become involved in a cancer organization that funds basic laboratory research. A number of these organizations host lectures given by their respective grant recipients and most of the lectures are given with a lay audience in mind. However, always try to understand the basic science rather than rely on lay "translations". If you do this, you will be adding another piece of information to your growing foundation of scientific knowledge.

There are several professional journals that one can subscribe to: *JNCI (Journal of the National Cancer Institute), Oncology, New England Journal of Medicine, Nature, American Journal of Human Genetics, Cancer, Endocrinology*, etc., plus popular press – *Scientific American*, etc. Many of these can be accessed online. The Library of Medicine can be accessed online (www.nlm.nih.gov) and has thousands of journals, articles and health related information. It is easy to access and easy to use.

Chapter 2

Resources

Accessing organizations that are involved in cancer research is quite easy in this day and age of websites and computers. There is a plethora of cancer organizations of which one can become a part. In addition, the Department of Defense (DoD) Congressionally Mandated Research Program includes advocates from cancer organizations in its research initiatives during the peer review process and on its Integration Panels. Members are selected based on criteria developed by DoD and a prerequisite is that one must be associated with a constituency so that multiple points of view are represented, not just one's personal cancer experience. Training is provided and participation welcomed. They have an extensive database of organizations and participants in their cancer program initiatives. Their website is: http://cdmrp.army.mil.

As mentioned earlier, the National Cancer Institute's Office of Liaison Activities has a listing of over 135 organizations whose missions are cancer related. You may begin by contacting one of the organizations listed either at the national level or in the local community in which you live, assuming that it is an affiliate of the national organization. Consumer Advocates in Research and Related Activities' (CARRA) website is http://la.cancer.gov/carra. Some of the major organizations one might begin with, several of which have local affiliates are:

American Cancer Society (ACS); www.cancer.org

National Breast Cancer Coalition (NBCC); www.nbcc.org

National Coalition of Cancer Survivorship (NCCS); www.canceradvocacy.org

Susan G. Komen Breast Cancer Foundation; www.komen.org

Y-Me National Breast Cancer Organization; www.y-me.org

Investigate and find the one that best suits your interests and needs.   Those that actually fund basic research have in some instances included advocates in the peer review process for grant applications, both nationally and locally if the local affiliate awards grants to researchers working in local institutional laboratories.

The Food and Drug Administration includes advocates as consumer representatives on Agency advisory committees to represent the consumer perspective on issues and actions before the various committees; serve as liaison between the committee and interested consumers, associations, coalitions, and consumer organizations; and facilitate dialogue with advisory committees on scientific issues that affect consumers. Consumer representatives must be able to analyze scientific data, understand research design, discuss benefits and risks, and evaluate the safety and efficacy of products under review (www.fda.gov). Consumer representatives must have an affiliation with or be an active participant in a community-based organization and can provide testimony before FDA committees.

Chapter 3

Avenues to Serve

There are many avenues in which an advocate can provide valuable service in the research arena:

Locally: Begin by becoming involved with a cancer organization that funds basic research. The Susan G. Komen Breast Cancer Foundation is dedicated to eradicating breast cancer as a life threatening disease and funds basic, translational and clinical research at the national level. Many of the grant recipients carry out their work in local communities that have teaching/research based medical centers. Many of these are designated by the National Cancer Institute as Comprehensive Cancer Centers and must meet rigorous criteria to be so designated. Become involved in the grant making process and team up with one of the grant recipients to learn about the specific research that he/she is investigating. Visit the laboratory; talk with the research associates about the scope of the research being conducted. Ask questions about their understanding of how this will impact cancer treatment or prevention. How soon will the particular research be available for utilization at the bedside? Ask them to explain any aspects that you do not understand. It has been the experience of the authors that scientists are enthusiastically willing to talk about their research and will often give you background reading from published papers. Once you have established your credibility as an informed and knowledgeable advocate, begin to give input from your perspective. Position yourself as an advocate resource, providing your thoughts and perspective. Take every opportunity presented to participate and do not be shy about requesting participation.

Nationally: Once involved at the local level, it is much easier to become involved at the national level in a variety of capacities. You may represent your organization as a representative to a variety of research symposia. National organizations are often invited to participate in government, pharmaceutical and foundation symposia related to breast cancer research as well as other issues related to the cancer experience. This is an opportunity for you to add to your basic scientific knowledge as well as keeping your ear to the ground for other opportunities for which you may volunteer your organization and yourself. The Department of Defense Congressionally Directed Medical Research Programs' Breast Cancer Research Program utilizes advocates from a large database of breast cancer organizations to participate in a two-tiered process of basic, translational and clinical research scientific review. A larger group of advocates is utilized in the peer review process; the first tier, and offer valuable input from the advocates' perspective of the science being presented. Advocates must be members of an organization involved in the breast cancer arena and undergo orientation and training to serve. Advocates who serve on the Integration Panel, the second tier of review, are generally leaders in their respective organizations and are selected based on experience, expertise and national representation. It is important to note that one experience leads to another and another and so on. We must stress here that any representation of your specific organization must be backed up with substantive knowledge and an ability to articulate issues of significance in breast cancer research in a thoughtful, objective, firm, yet respectful way.

Internationally: There is ever increasing interest from other countries in the model of advocate involvement in breast cancer research and indeed, international symposia that address current issues. Your organization may be included in one of these symposia – either as a

panelist, presenter or workshop leader – among the many opportunities. Again, the stress is upon credible, substantive articulation of issues.

Chapter 4

Participation as a Partner

Participation as a partner in research with scientists requires objectivity and a very strong sense of self. One must ascribe to the belief that the advocate's contribution to the scientific research process is critical and has a major impact upon national research directions and how that research is carried out. Participation is not about you and your specific experience with cancer, although such experience informs your contribution. It is about breast cancer for the general population. How quickly can we prevent or eliminate this disease?

Advocates become involved, as stated earlier, because breast cancer has touched their lives in a significant way. So much so, that they are compelled to "do something" to eradicate the disease or better yet, to prevent it. The avenue to eradication of current disease and prevention is initially through basic research. Advocates lend an air of urgency to the research at hand. The saving of lives depends upon the speed with which new discoveries are translated into clinical practice. Advocates posing a question or contributing an observation during scientific presentations underscores the need to stay focused on the goal. Extraneous avenues that have little contributory value, though fascinating, are less appealing. There are limited resources to get to the goal and it behooves everyone involved in the scientific process to use those resources as wisely as possible.

It is important to be knowledgeable regarding the research upon which you wish to make a comment and how that research will be carried out in the clinical setting. Is the research going to translate into a clinical trial? How soon? What are the ramifications for the participants of the trial? Is there drug development involved, a biologic, or is the research leading to a non-toxic or less invasive form of treatment or prevention? Are adequate patient safeguards being considered as the research progresses? Will patients be willing to participate in a trial if there are substantial biological side effects? Are informed consent documents considered early on in translating basic research to the clinical setting? Are they adequate to protect participants from harm?

As you can see from the comments in the previous paragraph, it is important to focus upon the end treatment early in laboratory research. Safe and effective treatments that work wonders in animal models will not always translate into the human cell environment. It may mean beginning again with a new hypothesis and scraping years of work because the original hypothesis has not been borne out in the laboratory. That is to be expected in basic research. Research findings advance the body of knowledge from which others may draw. Serendipitous events occur in the laboratory! A scientist working in a specific area may observe an unusual outcome in an experiment, note it, repeat it, and eureka, a scientific breakthrough is announced! All of these occurrences in the laboratory are necessary in order to arrive at the juncture for a clinical trial to be contemplated, designed and conducted.

Chapter 5

Rewards

Diving into basic breast cancer research has extraordinary rewards. It is an opportunity for you as an advocate to learn, to provide the advocates' perspective and to ultimately make a difference in the lives of those who are diagnosed with breast cancer. You, and/or your family member who may have experienced cancer personally will have a particular sensitivity to cancer research findings. Currently there is tremendous interest in finding a treatment or prevention that is non-toxic, one that does not have the plethora of side effects of today's standard treatments.

Prevention is more and more at the forefront of basic scientific research. Imagine interrupting the initiation of cancer with a non-toxic pill. Research is ongoing to do just that! One of the authors is an advocate on a grant looking at estrogen carcinogenesis, how to interrupt the estrogen metabolic pathway leading to cancer.

Both have authored books and/or articles in breast cancer research and treatment, participated in national and international panels, and are invited presenters at government hearings, and symposia, have given testimony before congressional bodies, served on research peer review panels, committees of Cooperative Research Groups such as CALGB, NSABP etc.. Each has had the opportunity to share her experience with cancer and the necessity of funding basic research with an intensity that will translate the findings in the laboratory to treatment at the bedside. Advocates help provide the focus and generate the impetus to move forward rapidly.

Advocates who are very involved with organizations focused on research and knowledgeable about current research are valuable resources in anticipating consumer issues in translating basic research into the clinical setting. They are adept at identifying issues in research design, informed consent documents, public communications, side effects, and recruitment to name but a few. Many have been participants in clinical trials and have come to appreciate the tremendous effort and dedication of scientists in solving the mysteries of this disease. They have indeed worked in partnership and value that partnership! One of the most heartbreaking experiences is to sit at the bedside of a loved one who has fought a valiant fight only to lose the battle just short of some magnificent discovery that, in the not too distant future, translates to treatment. It is too late for them, and such incidents further fuel the urgency that advocates feel in unlocking the keys to preventing cancer.

The ultimate reward for both scientists and advocates will be the prevention of cancer. Imagine cancer relegated to the category of preventable disease! It is on the horizon, and thus all scientific, advocate, and funding efforts must be focused to that end.

Chapter 6

Shared Experience

The authors have found different avenues to serve as an advocate and each has included a brief personal profile in hope that it will serve as a motivator to become involved. In addition more extensive vitae are included in the appendix to further demonstrate the variety of opportunities for involvement. Remember, what is required is the sincere interest and persistence!

Elizabeth A. Hart, President & CEO

HART INTERNATIONAL-Partnerships Advancing Women's Health Research

Multiple members of my family have died of cancer, the majority of whom were diagnosed with breast cancer. However, there were other cancers as well. Briefly, my two female first cousins – breast cancers (dead); mother-breast cancer (dead); sister-breast cancer (dead); oldest brother-throat and lung cancer (dead); brother-breast, kidney, colon, and liver cancer (survivor); and father-prostate cancer (dead). Both my daughter and I have had breast biopsies that fortunately to date have been benign. When my mother was diagnosed in 1984, it was the same year I became involved in the Susan G. Komen Breast Cancer Foundation and subsequently became Vice Chairman of Grants and finally Chairman and CEO. I was enormously interested in the research that Komen funded and along with a task force restructured the grant making process into a peer-reviewed system that allowed the organization to fund cutting edge research. The more I talked with scientists and learned about the avenues of research they were pursuing, the more I felt that the only way to eradicate the disease was to find out how cancer was initiated, proliferated, and metastasized. Research had the best chance to answer the questions that resonated each time I learned of a new diagnosis of cancer in my family and close friends. I immersed myself in laboratory breast cancer research, and have subsequently come to believe that cancer can be prevented. Research is more and more focused upon prevention initiatives. I am currently a participant with scientists on a Department of Defense Center of Excellence Grant that may well lead to prevention of breast cancer. The path I have traveled as a advocate has brought me through many of the finest research laboratories in the country and in contact with brilliant minds that I know hold to key to the prevention of this disease. I have lost many friends and family members along the way, some of them researchers, but their deaths have not been in vain. Each has added to the store of knowledge that will one day totally obliterate this disease. It cannot come to soon!

Elizabeth Hart can be reached via e-mail at ehart@hart-international.com

HART INTERNATIONAL was formed in 1995 by Ms. Hart specifically to work in partnership with scientists to advance women's health research, particularly breast cancer research at the basic scientific level.

Margaret Borwhat, Co-Founder & President

Women's Cancer Advocacy Network (WCAN)

As a survivor of cancer and currently battling a recurrence, I am indebted to basic research for offering hope for survival. Having been involved in the cancer arena since 1993 as a advocate, I focused attention on legal and ethical issues for breast cancer patients, serving in a variety of leadership roles culminating in the co- founding of WCAN, Women's Cancer Advocacy Network in 1997 and serving as its President.   It has been my passion to work with breast cancer researchers providing the advocate's perspective and helping facilitate the translation of basic research into the clinical setting, looking particularly at ethical issues of treatment impacting the lives of patients. WCAN is a source of accurate information and advocacy related to issues in breast cancer, and training.

Margaret Borwhat can be reached via e-mail at mborwhat@wcan.org

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APPENDIX

CURRICULUM VITAE

ELIZABETH A. HART, PRESIDENT & CEO

HART INTERNATIONAL

Brigham & Women's Hospital School of Nursing	Diploma	1963	Nursing	Boston, MA
George Washington University	BA	1971	Psych/Eng	Washington, DC
LeTourneau University		1999	MBA studies	Dallas, TX

PARTIAL LISTING

Ms. Hart is from a family with 5 first degree relatives with cancer: breast, prostate, kidney, throat & lung, colon and liver

1995-Present   President & CEO, HART INTERNATIONAL Mission: Develop Partnerships Advancing Women's Health Research in the area of cancer. Key focus is to facilitate/develop partnerships among advocates, organizations and funding sources to advance basic scientific research.

2001-Present   Appointed to Consumer Advocates in Research and Related Activities (CARRA) of the National Cancer Institute as Mentor. Reviewed and scored applications on Study Sections. Participants serve in a variety of capacities: setting research priorities, evaluating and developing information for public dissemination, identifying gaps in research and evaluating clinical trials from the patient advocate's perspective

2000-2003   Member, Data Safety & Monitoring Committee-International Breast MRI Consortium

1997-1999   National Cancer Policy Board – Initial Board Member- Identifying policy issues significant to cancer research and treatment on a national level. Providing expertise for publication and dissemination of board findings.

1996-Present   Member and Cancer Advocate to Cancer Cube – Collaborative Group of Scientists working on the etiology of breast & prostate cancer, variety of disciplines from sixteen institutions across the country. Responsible for advocate perspective into research agenda.

1994   Program Committee – Special Conference Industries Coalition Against Cancer

1993, Feb. 4   Testimony before the Institute of Medicine's Committee to advise the Department of Defense Breast Cancer Research Program

1993   Chairman, Komen Alliance: Komen Foundation, UT Southwestern Medical Center and Baylor University, a consortium working together on breast cancer research and clinical practice

1993-1994   National Chairman for Susan G. Komen Breast Cancer Foundation/NCI Regional Breast Cancer Leadership Summits – Major and Minor Summits to reach expanded and more diverse populations, with particular emphasis on minority populations

1993-1997   Consultant to Department of Defense (DoD Congressionally Directed Medical Research Programs (CDMRP) U.S. Army Breast Cancer Research Program (BCRP) providing expertise in program design, development and execution, review of proposals in multiple disciplines, service on initial Integration Panel, and subcommittees.

1995-1996   Member-Executive Secretaries Liaison Subcommittee, DoD BCRP

1994-1998   Member-Consumer Evaluation Subcommittee and Writing Group, DoD BCRP

1994-1995   Member-Executive Committee- Integration Panel, DoD BCRP

1995-2000   National Action Plan on Breast Cancer – Ensure Consumer Involvement Working Group

1995   CALGB Ad Hoc Committee on Policy for Genetic Research in Clinical Cancer Trial Patients

1995   NSABP Breast Cancer Prevention Trial Patient Advisory Committee

1995   NSABP BCPT Subcommittee for Clinical Center Performance Evaluation

1995   NCI SPORE Special Review Committee

1995   Industries Coalition Against Cancer Symposium – Panel Chairman –*Tying Cancer Education, Prevention, Screening to an Overall Wellness Program. April 1, 1995*

1995   RDOG Data Safety and Monitoring Board

1995, Feb.14   Testimony before FDA's Oncologic Drug Advisory Committee – "Consumers on ODAC"

1994-1995   Chairman & CEO, Susan G. Komen Breast Cancer Foundation – responsible for overall functioning of a national/international non profit organization with multiple affiliates whose mission is to eradicate breast cancer as a life threatening disease through research, education, treatment and screening. Under Hart

1994   NSABP BCPT Steering Committee

1994, June 7   Testimony before FDA's Oncologic Drug Advisory Committee "Resume Breast Cancer Prevention Trial"

1994   Program Committee and Dorothy Height Lifetime Achievement Awards Selection Committee – 5th Biennial Symposium on Minorities, the Medically Underserved & Cancer "Cultural Diversity, Public Policy and Survivorship"

1993   Chairman Elect – Board of Directors, Chairman National Advisory Board, Susan G. Komen Breast Cancer Foundation

1993, Oct.14   HHS Secretary's Conference to Establish a National Action Plan on Breast Cancer – Basic Research Working Group

1993, Oct. 21   Testimony before the Board of Scientific Counselors, Division of Cancer Prevention and Control National Cancer Institute – Screening Mammography

1993, Mar. 18   Testimony before the President's Cancer Panel's Special Commission on Breast Cancer - Komen/NCI Breast Cancer Leadership Summits

1991-1992   National Co-Chairman Regional Breast Cancer Leadership Summits – Co-chaired with member of the National Cancer Advisory Board/NCI, a series of breast cancer summits across the country targeting the leadership of corporations, health care providers and minority populations to increase breast cancer awareness and education efforts

PUBLICATIONS, MEDIA & SYPOSIA, HONORS (Partial Listing)

2006   Cavalieri, Ercole; Chakravarti, Dhubajyoti; Guttenplan, Joseph; **Hart, Elizabeth**; Ingle, James; Janko-wiak, Ryszard; Muti, Paola; Rogan, Eleanor; Russo, Jose; Santen, Richard; and Sutter, Thomas. *Catechol Estrogen Quinones as Initiators of Breast and Other Human Cancers. Implications for Biomarkers of Susceptibility and Cancer Prevention.* 2006 (BBA-Reviews on Cancer -In Press)

2002   Andejeski, Yvonne, M.D., Erica S. Breslau, Ph.D., M.P.H., **Elizabeth Hart, R.N**., Nigina Lythcott, Dr. P.H., Linda Alexander, Ph. D., Irene Rich, D.N.Sc.. Isabelle Bisceglio, Ph.D., Helene S. Smith, Ph.D., Fran M. Visco, Esq., and the U.S. Army Medeical research and Materiel Command Fiscal Year 1995 Breast Cancer Research Program Integration Panel. *Benefits and Drawbacks of Including Consumer Reviewers in the Scientific Merit Review of Breast Cancer Research.* Journal of Women's Health & Gender-Based Medicine, Volume 11, Number 2, 2002.

2000   **Hart, Elizabeth**, *Chapter 10 – Hope for Prevention: Perspective of the Cancer Advocate.* Journal of the National Institute Monograph,   Number 27, pages 157-159. 2000.

1999   National Cancer Policy Board – *Ensuring Quality Cancer Care.* National Academy Press, Washington, DC.

1998   Panelist – Women's Forum – "Menopause: Treatment Options for Women Surviving Breast Cancer or Concerned about Estrogen Replacement Therapy: June 23, 1998, Oschner Clinic, New Orleans, LA. Sponsored by the Hormone Foundation.

1998   Participant –Press Conference, "Menopause: Treatment Options for Women Surviving Breast Cancer or Concerned About Estrogen Replacement Therapy," The Endocrine Society 80th Annual Meeting, June 24, 1998; New Orleans, LA.

1998   Chairperson-Advocates' Perspective Panel – International Symposium, *Estrogens as Endogenous Carcinogens in the Breast and Prostate.* March 16-17, 1998. McLean, VA.

1999-1997   Member, Technical Program Committee, "Era of Hope" Symposium, USAMRMC Breast Cancer-Research Program, October 31-November 4, 1997

1995   Association of Community Cancer Centers' 21st Annual Meeting *ReVisioning Oncology.* Speaker and Panelist, High Tech and Patient Advocacy Panel, "State of the Art Medicine?"

1996   University of Texas Southwestern Medical Center: Speaker, Breast Care: Advances & Update for Primary Care Physicians: A Didactic Skills Symposium, "Physician-Patient: Partners in Health", July 15, 1995

1995   Executive Producer "For Women's Lives: Dialogues on Breast Cancer" PBS film aired in Spring of 1996. Finalist for Best of Category – American Medical Association's International Film Competition, November 1996

1994   Opinion Editorial, "Peace dividend shows up in medicine", Dallas Morning News, November 14,

1994 -July   Participant "New Frontiers in Breast Cancer Imaging and Early Detection" Symposium sponsored by the Office of Women's Health, NCI, and the Congressional Caucus For Women's Issues, Washington, D.C.

<div align="center">**CURRICULUM VITAE**</div>

MARGARET J. BORWHAT, PRESIDENT

WOMAN'S CANCER ADVOCACY NETWORK

Russell Sage College	B.A.	1975	Psychology	New York
University of Maine at Orono	Masters Studies	1976	Social Psychology	New York
University of New York, Albany	MA/Ph.D.Studies	1981	Social Psychology	New York
	(Completed Coursework)			

<div align="center">**PARTIAL LISTING**</div>

CONTINUING EDUCATION

University of Virginia 1991-93, completed all courses required to sit for the CPA Exam. Research in International Accounting. 1995, Course in Strategic Planning for Non-profits.

Center for Clinical Bioethics, Georgetown University Medical Center, March 2000. Completed 14-hour workshop, Research Ethics: Institutional Review Board Responsibilities and Related Competencies.

Public Responsibilities in Medicine and Research (PRIM&R) at Tufts University School of Medicine, May 18-19, 2001. Completed 16-hour workshop, Promoting Responsible Conduct of Research: Policies, Challenges, and Opportunities.

Kennedy Institute of Ethics, Georgetown University, June 5-10, 2001. Completed Intensive Bioethics Course, XXVII, an in-depth study of major topics and contemporary challenges in health care and research ethics.

RELAVANT ADVOCACY WORK AND ORGANIZATION EXPERIENCE

Virginia Breast Cancer Foundation (VBCF) President, June 199-96, Vice President 1994-95, Legislative Chair 1993-95, Board Member 1993 -1997.

National Breast Cancer Coalition (NBCC) 1993-95, State Coordinator, served as liaison between NBCC and BVCF. Board Member, 1995, Completed Project Lead, 1996 – training program on the science of breast cancer for advocates.

Hospice of the Piedmont, Charlottesville.VA 1995-96 Volunteer

National Patient Advocate Foundation, June 1996 to June 1999, Legislative Liaison, Researcher, and Board Member. Researched 50 states' legislative bills and resolutions related to cancer and health care insurance. Formulated and helped implement legislative initiatives, conducted research, developed web page, and assisted in fundraising, establishing and maintaining databases.

Patient Advocate Foundation, June 1996 to June 1999. Board of Directors. Provided education and pro bono

legal counseling to cancer patients concerning managed care, insurance, and financial issues.

Legal Information Network for Cancer (LINC) T.C. Williams School of Law, University of Richmond, October 1997 to December 1998, Advisory Board, an organization of volunteers committed to helping patients and their families resolve non-medical issues arising from the diagnosis or treatment of cancer.

JAUNT January 1998 to 2000, Secretary, Board of Directors and Finance Committee, Virginia's Planning District Ten, providing services for the general public, human service agency clients, the elderly and people with disabilities.

Women's Cancer Advocacy Network (WCAN) 1997 to present, Co-founder and President, work with researchers and clinicians to provide consumer advocate perspective and to facilitate translation of research to clinical setting. Served on Advocate Core for SPORE projects and development of educational training modules for advocates. Public speaking and outreach, e.g., Breast Cancer Patient's Perspective presented at Virginia Commonwealth University for social work graduate students; and Internet Support Group Participation presented at the University of Virginia for medical staff and public. Development and distribution of materials for advocates including Department of Defense Breast Cancer Research Program Meeting, Era of Hope summary.

CONSULTATION AND SPECIAL PROJECTS

Consumer Reviewer of Grant Proposals for Department of Defense Breast Cancer Research Program 1995, Supplemental Grants, 1996; Endocrinology panel, and review of process; 1998, Molecular genetics panel.

Commonwealth of Virginia General Assembly Study Group on Patient Information 1996, Provided consumer perspective on legislative study group regarding informed consent and patient information.

Workshop Conference on Treatment of Estrogen Deficiency Symptoms in Women Surviving Breast Cancer. Served as patient advocate in the organization and participation of consumer advocates at an international conference held in September, 1997.

Georgetown University, Lombardi Cancer Center (LCC) June 1996 to present. Consumer advocate for Department of Defense Research and Specialized Program of Research Excellence (SPORE) in Breast Cancer Conducted at LCC.

Cancer and Leukemia Group B (CALGB) January 1999-2003, Consumer advocate on the Breast Committee, Patient Issues Committee, Clinical Economics Subcommittee, and Patient Advocate Working Group.

National Action Plan on Breast Cancer, May 1999, Consumer Involvement Working Group member studying existing consumer representation in a variety of breast cancer research and programs, and identifying areas for increased involvement.

Cancer Genetics Network (CGN, Bioethics Working Group, June 1999-2003, National Cancer Institute

Sponsored infrastructure to support genetics research. Contributed article for Mid-Atlantic Cancer Genetics Network, Spring 2000, and the Northwest Cancer Genetics Network, Spring/Summer, 2000.

Susan G. Komen Breast Cancer Foundation 1999, 2000, and 2001, Consumer reviewer for grant proposals, tumor biology section. Selected "Local Hero" for Komen's Drive for the Cure.

Office of Liaison Activities, March 2000, appointed to serve on the Advocate Committee to the Working Group on Liaison to Advocacy and Voluntary Organizations.

National Human Research Protections Advisory Committee (HHRPAC), Third Parties Working Group January 2001 to present. Provides advice and recommendations to the Secretary of HHS, Assistant Secretary for Health (ASH), the Director, Office for Human Research Protections (OHRP), and other departmental officials on issues and topics pertaining to the protection of human research subjects.

National Human Research Protections Advisory Committee (NHRPAC) January 2001 to September 2002. Appointed to serve a three-year term, member of Third Party/Genetics Working Group, and Informed Consent Working Group. Provides advice and recommendations to the Secretary of hhs, Assistant Secretary for Health (ASH), the Director, Office for Human Research Protections (OHRP), and other departmental officials on issues and topics pertaining to the protection of human research subjects.

National Cancer Institute Central Institutional Review Board 2003-2005, IRB review of NCI sponsored multi-site trials at the national level before the protocol is distributed to local investigators.

Cancer Biomedical Informatics Grid, caBIG, patient advocate, 2004-2005, a voluntary network or grid connecting individuals and institutions to enable the sharing of data and tools, creating a World Wide Web of cancer research under the leadership of National Cancer Institute's Center for Bioinformatics.

PUBLICATIONS

Menopause: Treatment Options for Women Surviving Breast Cancer or Concerned about Extrogen Replacement Therapy. Santen, Richard J., M.D.; Borwhat, Margaret, and Gleason, Sarah, The Hormone Foundation, 1998. Revised 1999, *Managing Menopause: A Change for the Better.*

"Managing accrual in cooperative group clinical trials:, Demmy, TL, Yasco, JM, Collyar, DE, Katz, ML, Krasnov, CL, Borwhat, MJ, Battershell, A, George, SL. *Journal of Clinical Oncology.* August 1, 2004, 2997-3002.